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**TITLE: Prophylactic Administration of CN-105 Confers Neuroprotection
Against Acute Brain Injury**

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- 1. INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

We have previously established that the pentapeptide CN-105 (Ac-VSRRR-COOH) improves functional and outcome and reduces histological injury in a murine model of traumatic brain injury (closed head injury caused by fluid percussion against the intact skull). The major goal of this project is to establish whether this neuroprotective pentapeptide would be effective in mitigating secondary neuronal injury and improving functional outcomes when administered prior to closed head injury, as this would be a potentially viable strategy to reduce morbidity of traumatic brain injury associated with combat. As described in the statement of work, during the initial year of this proposal, we have tested the efficacy of CN-105 when administered prophylactically in both a moderate single head injury, and paradigm of milder head injuries occurring on a weekly basis over 5 weeks. In addition, we performed the initial work comparing the bioavailability and pharmacokinetics between intravenous, intraperitoneal, intranasal, and oral modes of administration.

- 2. KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Neuroprotection, closed head injury, murine models, neuroinflammation, functional recovery, peptide, therapeutic, glia

- 3. ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

We have accomplished the major objectives, as defined in the original SOW. As outlined in the NCE, in the coming quarters, we plan to reproduce the short term (TBI) and long term studies (repetitive TBI) in preparation for submitting manuscript and national presentation. The major objectives, as stated in the approved SOW were:

Objective 1: Does prophylactic administration of CN-105 mitigate cellular injury and neurocognitive decline in a murine model of severe TBI? What is the optimal prophylactic therapeutic window of CN-105?

Objective 2: Does prophylactic administration of CN-105 mitigate cellular injury and neurocognitive decline in a murine model of mild, repetitive TBI?

Objective 3: What is the pharmacokinetic profile of CN-105 following intranasal and intraperitoneal administration?

What was accomplished under these goals?

Introduction: We have previously established that the pentapeptide CN-105 (Ac-VSRRR-COOH) improves functional and outcome and reduces histological injury in a murine model of traumatic brain injury (closed head injury). The major goal of this project was to establish whether this neuroprotective pentapeptide would be effective in mitigating secondary neuronal injury and improving functional outcomes when administered prior to closed head injury, as this would be a potentially viable strategy to reduce morbidity of traumatic brain injury associated with combat. As described in the statement of work, we tested the efficacy of CN-105 when administered prophylactically in both a moderate single head injury, and paradigm of repetitive milder head injuries occurring on a weekly basis over 5 weeks. We compared the bioavailability and pharmacokinetics between intravenous, intraperitoneal, intranasal, and oral mode of administration.

Objective 1: Does prophylactic administration of CN-105 mitigate cellular injury and neurocognitive decline in a murine model of severe TBI? What is the optimal prophylactic therapeutic window of CN-105?

Methods: To accomplish this objective, we utilized our model of closed head injury previously established in our lab. This murine model involves pneumatic impact against the closed skull with serial functional assessments of vestibulomotor (Rotorod latency) and cognitive (Morris water maze testing) performance.

Briefly, 12-14 week-old C57Bl/6J male mice (Jackson Laboratories, Bar Harbor, ME) will be used for this model. The trachea is intubated after anesthesia induction with 4.6% isoflurane and the lungs are mechanically ventilated with 1.6% isoflurane in 30% O₂/70% N₂. Core body temperature is maintained at 37°C through a rectal probe. The animal is secured in a stereotactic device, the scalp is incised and the skull exposed. A concave 3-mm metallic disc is adhered to the skull immediately caudal to bregma. A 2.0-mm diameter pneumatic impactor (Air-Power Inc., High Point, NC) is used to deliver a single midline impact to the center of the disc surface. The impactor is discharged at 6.8 ± 0.2 m/second with a head displacement of 3 mm. After impact, the animals are allowed to recover spontaneous ventilation and then the tracheas are extubated. Following recovery, mice are allowed free access to food and water.

In all studies, mice are randomized to treatment or vehicle, and both the surgeon perform in the TBI procedure and personnel performing behavioral assessments are blinded to treatment allocation. Animals are placed in a restrainer (Harvard Apparatus, Holliston, MA), and a single intravenous dose of drug will be administered by tail vein in a volume of 100 μ L. Vehicle treated animals receive intravenous injection of 100 μ L of normal saline at the same time points. Animals will be assigned to treatment group by a coded study identification number after injury using a paper randomization protocol. A block randomization scheme is used so that an equal number of animals are randomized to each of the treatment groups during concurrent experiments.

Clinically relevant behavioral assessments are performed on all animals. This includes tests of vestibulomotor function (Rotorod latency) and cognition (Morris water Maze). An automated Rotarod (Ugo Basile, Comerio, Italy) will be used to assess vestibulomotor function (Hamm et al., 1994). On the day prior to injury, mice (n=11-12 mice per group) will undergo one training trial at an accelerating rotational speed (4-40 rpm) for at least 200 seconds and then three additional test trials with the same accelerating rotational speed. The average time to fall from the rotating cylinder in the test trials is recorded as baseline latency. On days 1-7, 14, and 21 post-injury, the mice will have three consecutive daily trials with accelerating rotational speed (inter-trial interval = 15 minutes). The average latency to fall from the rod is recorded. Mice unable to grasp the rotating rod are given a latency value of 0 seconds. As described previously (Morris, et al. 1984), the Morris Water Maze assesses spatial learning and memory by testing the ability of mice to locate a submerged platform. The mice are placed in a pool (105 cm diameter) filled with liquid and allowed up to 90 seconds to locate the submerged platform. The mice will perform four trials/day for 4 consecutive days (inter-trial interval = 30 min). The mice are introduced in varying quadrants of the pool for each trial but the location of the platform never varies. The latency to locate the platform will be recorded, and the 4 trials per day will be averaged. Mice will be tested on days 28-31 post-injury (n=11-12 mice per group).

Based on the short half-life of CN-105, in the initial series of experiments, CN-105 compound was administered intravenously 10 and 20 minutes prior to injury, and this was compared to post-injury treatment. As demonstrated in **Figure 1A-C** prophylactic treatment improved outcome, although not to the extent of post-treatment. As defined in the grant proposal, once we established efficacy at 10 and 20 minutes prior to injury, we next tested the hypothesis that we could extend the therapeutic window of CN-105 prophylactic pre-administration to 30 and 60 minutes prior to closed head injury. As before, CN-105 was administered by intravenous administration at a dose of 0.05 mg/kg at 30 minutes (n=14 animals) or 60 minutes at a dose of 0.05 mg/kg (n=15) and compared to vehicle treated TBI animals (n=14) and animals that underwent sham procedure (n=15). There was a statistically significant and durable improvement in functional performance when animals were treated with 0.05 mg/kg intravenously 30 minutes prior to the induction of head injury ($p=0.0087$ as compared to vehicle treated TBI animals; **Figure 2**). However, this neuroprotection was greatly diminished when drug was administered at the same dose and route 60 minutes after TBI. This may be due to the relatively short plasma half life of CN-105 (measured at 30 minutes in rodent models)

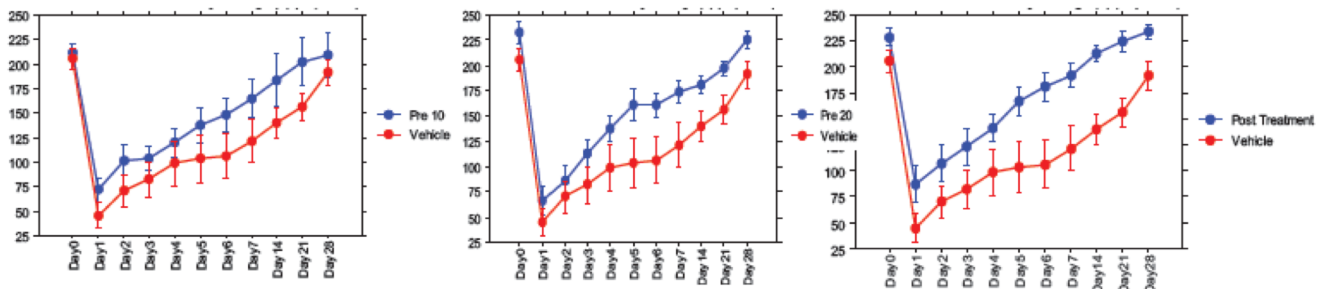
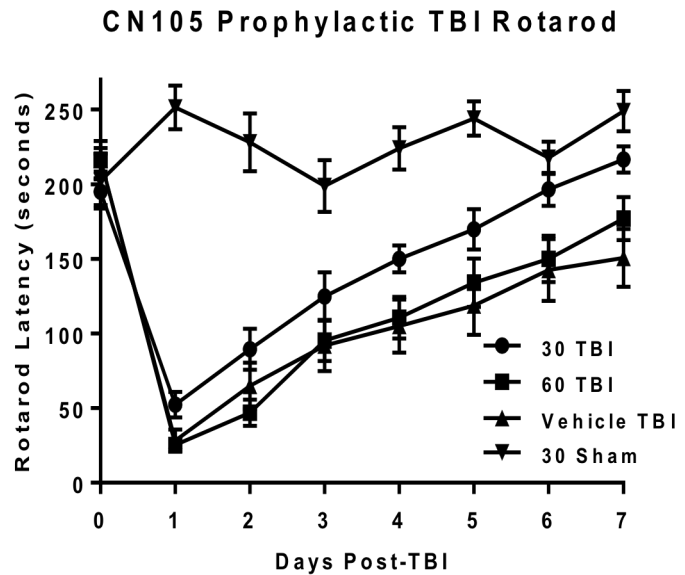


Figure 1A) 10-12 week old Male C57-BL6 were randomized to receive either saline vehicle (n=14) or 0.05 mg/kg CN-105 delivered intravenously by tail vein (n=11); there was a trend towards improved performance after administration of CN-105 ($p=0.15$ as assessed by two level ANOVA). **B)** Treatment at 20 minutes prior to TBI (n=13 animals randomized to 0.05 mg/kg CN-105) demonstrated statistically significant and durable improvement in Rotorod performance ($p=0.2$ as assessed by two level ANOVA) as compared to vehicle treated animals (n=14). **C)** Although pretreatment was associated with benefit, functional improvement was greatest when animals were treated with CN-105 0.05 mg/kg post-treatment (n= 14 animals post-treatment; as compared to vehicle, $p=0.0054$).

Figure 2: 10-12 week old male C57-BL6 were randomized to receive either sham procedure (inverted triangles; n=15); and closed head injury preceded by saline vehicle (n=14; filled upright triangles); and 0.05 mg/kg CN-105 by intravenous injection either 30 minutes prior to TBI (n=14, filled circles); or 60 minutes prior to TBI (n=15, filled squares). There was a statistically significant and durable improvement in Rotorod latency when animals were treated with CN-105 minutes 30 minutes after injury as compared to vehicle treated TBI animals ($p=0.0087$ as assessed by two factor ANOVA). There was diminished efficacy, but still a trend for animals treated with CN-105 at 60 minutes prior to TBI to have improved rotorod performance as compared to vehicle treatment. Statistical assessments were made with 2 factor ANOVA with Sheffe post-hoc correction for multiple comparisons.



We next performed an follow-up series of experiments re-testing prophylactic efficacy at 3 and 6 hours prior to TBI. To test this, 10-12 week old male C57-BL/6 mice were randomized to receive vehicle (n=15); CN-105 at a dosage of 0.2 mg/kg delivered intravenously by tail vein injection in a volume of 100 ul at 3 hours prior to injury (n=15); CN-105 at a dosage of 0.2 mg/kg delivered intravenously by tail vein injection in a volume of 100 ul at 6 hours prior to closed head injury (n=15). As outlined in **Figure 3**, there was no statistically significant improvement at either 3 hour or 6 hour pre-incubation prior to injury

Thus, our data suggested that, although, as proof or principle, CN-105 was effective when administered prior to closed head injury, the peptide had to be present in sufficient concentrations at the time of injury to prevent pathological glial activation, and the relatively short half life (measured as 29 minutes) limited this therapeutic window to < 60 minutes. **To overcome this limitation**, we next performed an additional series of experiments, where higher dose of CN-105 (1 mg/kg) was administered intra-peritoneally to maximize drug availability. In this paradigm, we did demonstrate durable effect through 14 days tested, functional improvements most robust up to 14 days post-injury; (**Figure 4**)

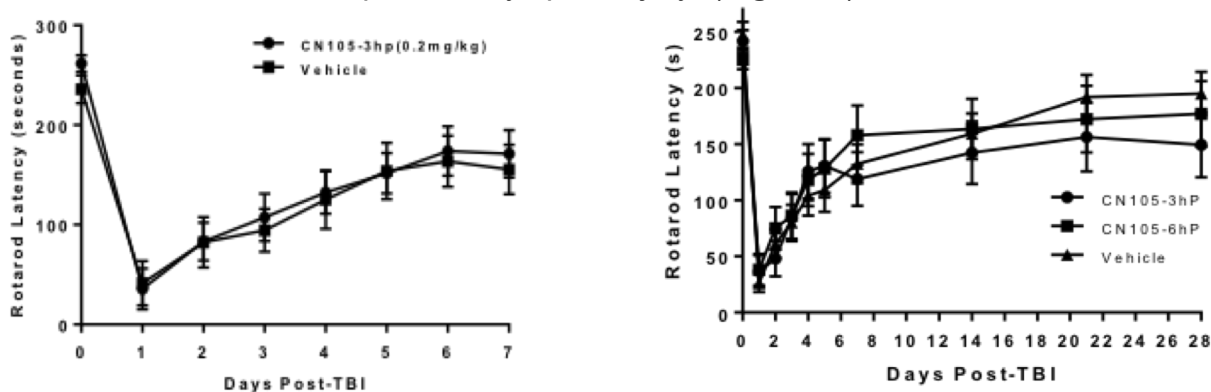


Figure 3: Pre-administration of intravenously administered CN-105 at a dose of 0.2 mg/kg did not affect functional outcome as assessed by serial Rotorod latency when administered at 3 hours or 6 hours (**B**) prior to TBI at a dose of 0.2 mg/kg (drug administered intravenously by tail vein in 100 ul sterile isotonic saline)

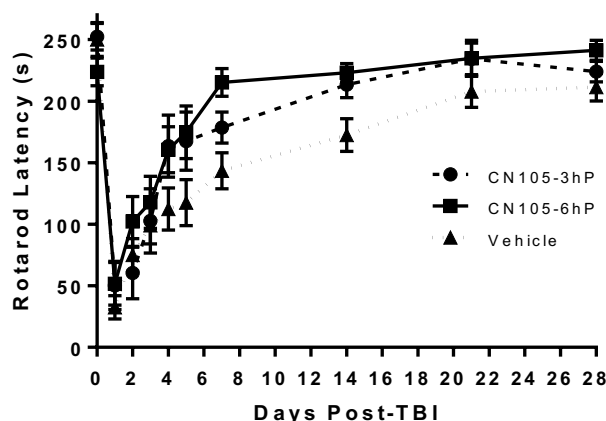


Figure 4: Pre-administration of CN-105 at a dose of 1.0 mg/kg improved functional outcome as assessed by serial at up to 14 days. Animals were randomized to receive 1 mg/kg CN-105 at 3 hours prior to injury (n=10); 6 hours prior to injury (n=11), or vehicle at 3 hours prior to injury n=12). Analysis was performed by 2 factor ANOVA (stratifying by treatment group and time), and conducted for the first 14 days) (* $p < 0.05$)

In summary, our data suggests as proof of principle, that CN-105 has efficacy when used prophylactically prior to traumatic brain injury. The therapeutic window from drug administration to injury is dependent on maintaining sufficient concentrations of CN-105 at the time of injury. This is a function of the amount and mode of administration of drug. Of note, the pharmacokinetics of CN-105 is a function of species; while rodent has a serum half life of 30 minutes, in phase 1 testing, serum half life in human subjects was approximately 3.5 hours (Guptill et al., included as Appendix), which may make this more amenable to clinical use. However, to demonstrate proof of principle, in Objective 3, we define and compare pharmacokinetics associated with intranasal, oral, and intraperitoneal modes of administration with intravenous administration to assess alternative routes of delivery.

Objective 2: Does prophylactic administration of CN-105 mitigate cellular injury and neurocognitive decline in a murine model of mild, repetitive TBI?

Our second research objective was to evaluate efficacy of CN-105 in a model of mild-moderate repeat TBI, occurring at weekly intervals, for a total of five injuries. As described in the original proposal, an additional aim was to evaluate whether prophylactic administration of CN-105 would mitigate cellular injury and neurocognitive decline in a murine model of mild, repetitive TBI. We created the rTBI model. By reducing the displacement of the pneumatic impact, we have titrated the closed head injury to recreate a subclinical concussive force. Although this mild injury does not initially result in quantifiable vestibulomotor deficits, successive impacts result in cumulative functional and histological injury (Figure 7). This model recapitulates the clinical situation of repetitive mild TBI causing chronic neurocognitive deficits (chronic traumatic encephalopathy). The rTBI mice receive low displacement TBI injuries (rTBI mice) on Days 1, 7, 14, 21 and 31 for a total of 5 injuries.

In our initial experiments, two groups of male 12 week old C57-BL/6 mice were randomized to receive either 0.5 mg/kg CN-105 delivered by intraperitoneal injection performed 30 minutes prior to and 72 hours following each mild TBI. Serial Rotarod assessment was performed one day prior to and following the mild TBI. Both placebo and CN-105-treated groups demonstrated acute decline after each injury. However, by day 38, the ApoE-treated group had significantly better motor outcomes ($p = 0.015$; two factor ANOVA) compared to the placebo group (**Figure 5A**). Comparison of groups over time demonstrated that the ApoE-group's motor performance was significantly different over time. We also conducted a MWM 1 week post the final TBI (TBI 4). Cognitive function as assessed by the Morris water maze did not show significant difference between placebo and treatment groups (Figure 5B).

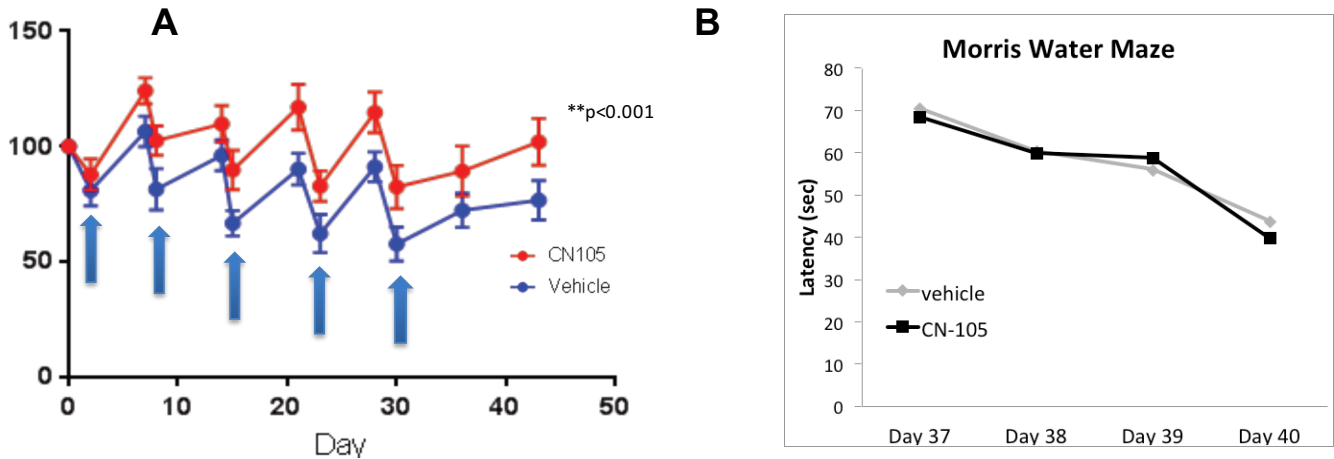


Figure 5A: 10 week old male C57-BL/6 animals were randomized to receive vehicle or 0.5 mg CN-105 by i.p injection 5/week. X 4 weeks (blue animals represent times of injury) Once/week animals receive a traumatic brain injury by pneumatic impactor with displacement of 2.5 mm, velocity of 6.8 m/sec. All animals tested serially to assess Rotarod displacement (initial value designated as 100%). CN-105 Treated animals had significantly better performance at all points tested ($p < 0.001$, by two factor ANOVA). **Figure 5B:** In Morris Water Maze assessment of cognitive performance, there was no significant difference in latency between CN-105 and vehicle treated animals. This data is for representative experiment, which was performed twice.

We next performed histology to assess whether the chronic treatment with CN-105 could reduce the long term histological injury associated with chronic traumatic encephalopathy associated with multiple repetitive head injuries. We found that, consistent with their improved functional performance at 90 days, animals with repetitive TBI protocol had a reduction in neuronal injury, quantified by unbiased stereology as spheroids in optic tract (**Figure 7**).

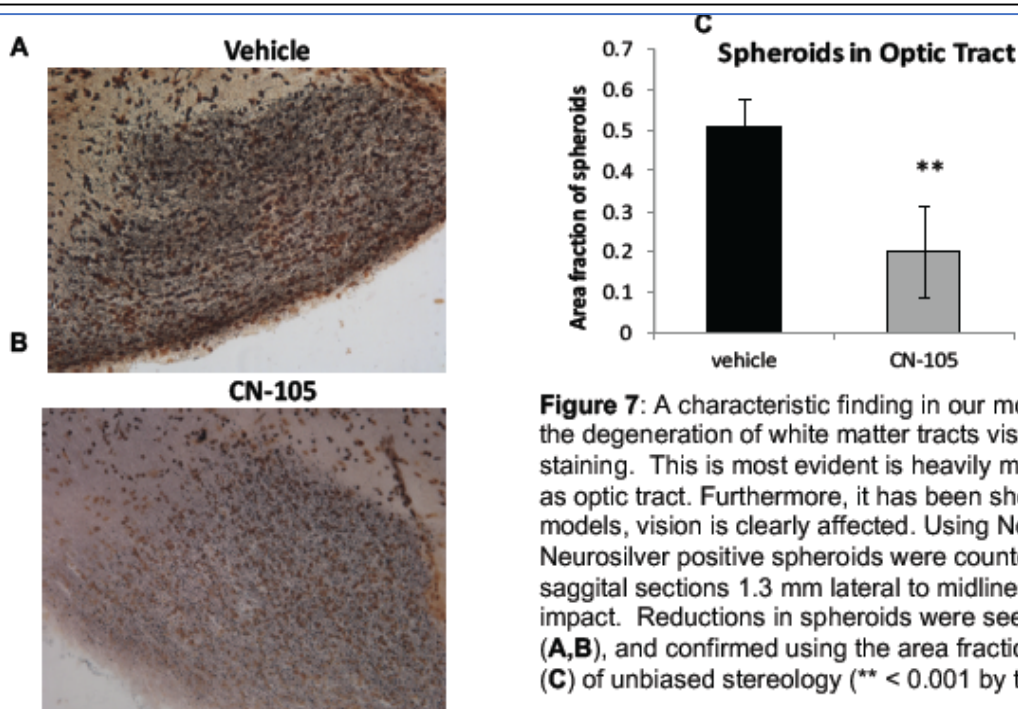
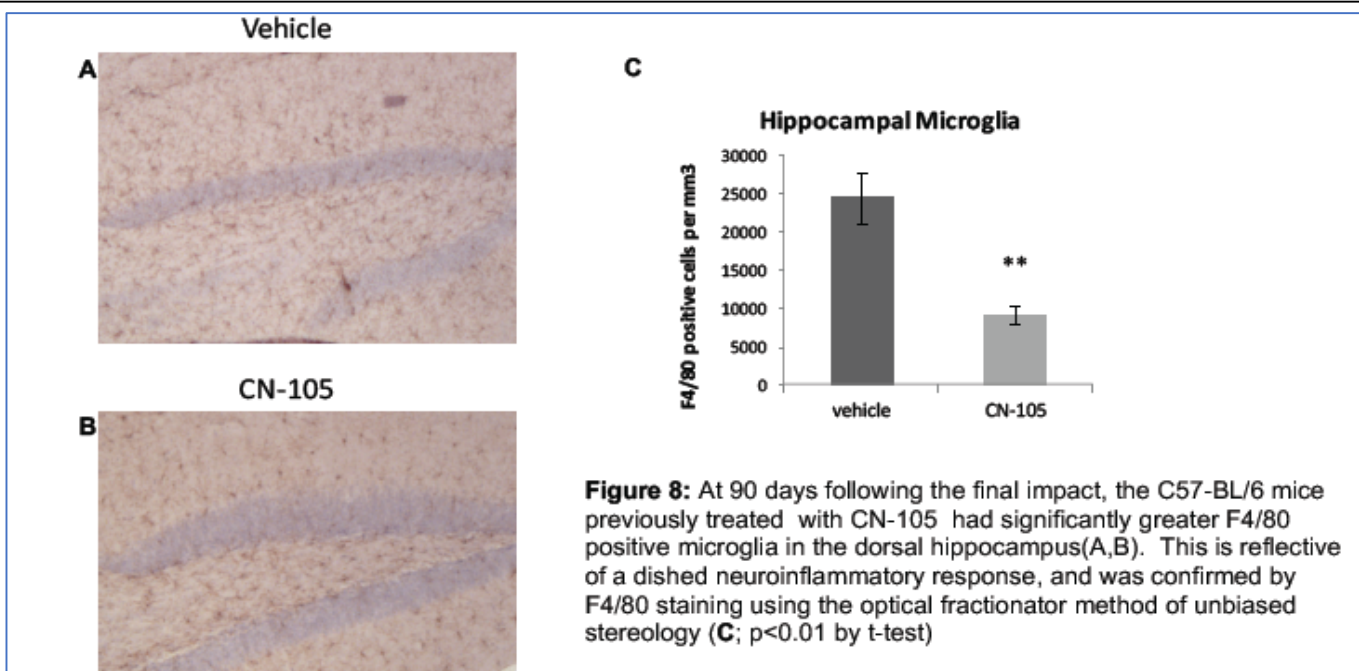


Figure 7: A characteristic finding in our model of repetitive TBI is the degeneration of white matter tracts visualized by Neurosilver staining. This is most evident in heavily myelinated tracts such as optic tract. Furthermore, it has been shown that in rodent models, vision is clearly affected. Using Neurosilver stain, Neurosilver positive spheroids were counted in the optic tract in sagittal sections 1.3 mm lateral to midline 90 days after last impact. Reductions in spheroids were seen in treated animals (**A,B**), and confirmed using the area fractionator method (**C**) of unbiased stereology ($** < 0.001$ by t-test).

Another chronic histological finding that is observed in this model of repetitive TBI is diffuse microgliosis, which is often pronounced in the hippocampus. To assess whether CN-105 influenced the development of microgliosis, we performed unbiased formal stereology to examine F4/80 positive neurons (this stain does not differentiate intrinsic microglia from hematogenously recruited monocytes, but does reflect the CNS inflammatory response). We found that treatment with CN-105 significantly reduced microgliosis 90 days after injury (**Fig 8**)



Objective 3: What is the pharmacokinetic profile of CN-105 following intranasal and intraperitoneal administration?

Research Objective 3 was designed to compare the pharmacokinetics and feasibility of different routes of administration of CN-105. This is important to establish whether there may be alternative noninvasive methods of drug delivery in the clinical setting (for example, intranasal), and also to optimize the prophylactic experiments.

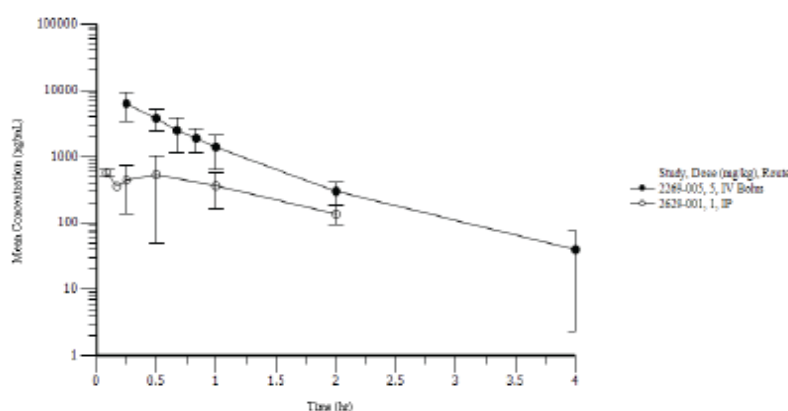
As outlined in the original proposal, briefly, 1 mg/kg CN-105 was administered as a single dose by intranasal (IN; dose volume 40 microliter/nares), intraperitoneal (IP), and oral (PO) dosing in cannulated 13-16 week old male Sprague-Dawley rats (n=3/group).

Pharmacokinetic (PK) samples were collected by sublingual vein at approximately 0.083, 0.167, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours post-dose. Aside from the IV formulation, CN-105 was only measurable in peripheral blood at 0.083 (5 min) hours postdose for animals in Group 2 (IN administration of CN-105) and all CN-105 plasma concentration were BLQ (< 25 ng/mL) for animals after PO administration of CN-105; therefore, exposure ratios between the different routes of administration could not be calculated (no AUC determined after IN or PO administration). Following a single IP administration of 1 mg/kg CN-105, mean C_{max} and AUC_{0-24hr} values for CN-105 were 725 ng/mL and 830 hr*ng/mL, respectively **Figure 9**. These results are summarized in **Table 1** and **Figure 9**.

Table 1: CN-105 Pharmacokinetics following IP, IN, and PO Delivery

Group	Dose (mg/kg)	Route	Subject	C _{max} (ng/mL)	C _{max} /Dose (ng*mg/mL/mg)	T _{max} (hr)	T _{1/2} (hr)	AUC _{Tmax} (hr*ng/mL)	AUC _(0-24hr) (hr*ng/mL)	AUC _(0-24hr) /Dose (hr*ng/mL/mg)	T _{1/2} (hr)	AUC _{INF} (hr*ng/mL)	AUC _{INF} /Dose (hr*ng/mL/mg)
1	1	Intraperitoneal	101	1040	1040	0.5	2	1180	1370	1370	NA ^a	NA ^a	NA ^a
			102	507	507	0.083	2	515	629	629	0.739	632	632
			103	628	628	0.083	2	386	495	495	NA ^a	NA ^a	NA ^a
2	1	Intranasal	104	2790	2790	0.083	0.083	NA ^b	NA ^b	NA ^b	NA ^a	NA ^a	NA ^a
			105	145	145	0.083	0.083	NA ^b	NA ^b	NA ^b	NA ^a	NA ^a	NA ^a
			106	307	307	0.083	0.083	NA ^b	NA ^b	NA ^b	NA ^a	NA ^a	NA ^a
3	1	PO	107 ^a	0.00	NA	NA	NA	NA	NA	NA	NA	NA	NA
			108 ^a	0.00	NA	NA	NA	NA	NA	NA	NA	NA	NA
			109 ^a	0.00	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA - Not applicable
^a All CN-105 plasma concentrations were BLQ (< 25 ng/mL)
^b AUC not reported due to less than three consecutive quantifiable concentrations
^c Secondary parameters (T_{1/2} and AUC_{INF}) not reported due to insufficient plasma concentration-time data
^d Secondary parameters (T_{1/2} and AUC_{INF}) not reported due to an adjusted R² less than 0.9

**Figure 9: Mean +/- SD plasma concentration-time profile following a single intraperitoneal administration of CN-105 at a dosage of 1 mg/kg in male Sprague-Dawley rats**

Based on this pharmacokinetic information demonstrating a more prolonged half life associated with i.p administration as opposed to i.v. route of administration (intraperitoneal T_{1/2} 0.74 hours vs. intravenous T_{1/2} 0.49 hours), we next optimized the prophylactic administration dosing paradigm described in Objective 1 by administering intravenous and intraperitoneal administration simultaneously at 3 and 6 hours prior to subjecting mice to moderate-severe closed head injury paradigm. Male 10-12 week old C57-BL6 mice were randomized into one of three groups: animals pretreated 3 hours prior to TBI with a combination of CN-105 0.1 mg/kg i.v. plus 0.5 mg/kg i.p (n=14); animals pretreated 6 hours prior to TBI with a combination of CN-105 0.1 mg/kg i.v. plus 0.5 mg/kg i.p (n=14); animals pretreated 3 and 6 hours prior to TBI with a combination of 100 microliters vehicle i.v. plus 0.5 mg/kg i.p (n=14). Serial Rotorod was performed daily for days 1-7 post-injury, and weekly until Day 28. With this optimized treatment regimen, we were able to observe a significant improvement in animals pretreated at 3 hours prior to injury (p<0.01 at day 28; **Figure 10**)

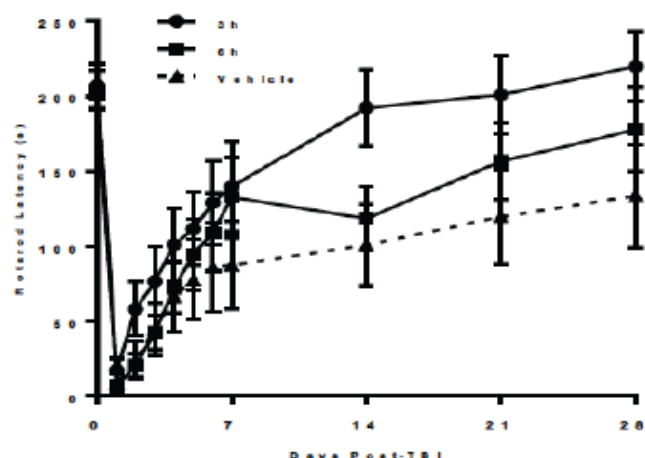


Figure 10: C57-BL/6 male 12 week old mice were treated with a combination of 0.5 mg/kg i.p. and 0.1 mg/kg i.v CN-105 or i.p and i.v vehicle at 3 hours and 6 hours prior to moderate to severe closed head injury. In the the 3 hour pre-treatment group, there was functional benefit, as defined by improved vestibulomotor function (Rotorod latency) for animals pre-treated 3 hours prior to injury ($p < 0.01$ by ANOVA).

These results demonstrate that, in theory, pretreatment with CN-105 could reduce functional deficits associated with subsequent head injury as long as dosing can be optimized so that there are adequate drug concentrations at the time of injury. It is important to note that, although the serum half-life of CN-105 is 29 minutes in murine model, the half life is 3.5 hours in humans (the full pharmacokinetic profile in clinical use is included as Appendix). This would suggest that chronic pretreatment may be a viable strategy for military at risk for TBI.

In summary, our results suggest that CN-105 reduces functional deficits and histological evidence of injury when administered in prophylactic fashion in both a severe acute closed head injury model and a chronic repetitive mild closed head injury model. The time window for efficacy is a function of concentration of CN-105 at the time of injury, which is influenced by the mode of delivery and amount of drug administered. Although we do not find evidence of bioavailability when administered via an intranasal route or oral route, the serum half life in humans is 3.5 hours, which is considerably longer than in rodents (30 minutes) suggesting feasibility in a clinical population. Of note, our preliminary data demonstrates that chronic administration reduces cumulative functional deficit associated with repetitive head injury, as well as long term structural changes. As part of the recently approved no cost extension, we are repeating these functional and histological results, which would have potential for clinical translation in a warrior population at risk for multiple TBI and progressive symptoms. As a note, in the Portfolio in Progress session, it was suggested that we also assess efficacy of CN-105 in the more traditional context of post-injury delivery. This would have particular benefit in the military setting, given tolerability, and the fact that treatment reduces progression of tissue injury caused by inflammatory mechanisms, which may extend the window by which more definitive treatments for intracranial hypertension may be initiated.

As suggested in the Portfolio in Progress review, we have also completed additional experiments evaluating more traditional (post-injury) dosing of CN-105 on functional and histological endpoints in a closed head injury model. This work was recently completed is in press, and included in the Appendix.

What opportunities for training and professional development has the project provided?

Nothing to report.

This project was not intended to provide training and professional development opportunities.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report. As noted below, in the coming year, during the period of no-cost extension, our intent will be to replicate and publish this work, as well as present this data at a national meeting, such as the Society for Neurocritical Care.

What do you plan to do during the next reporting period to accomplish the goals?

This is the final report for this project's initial funding period. However, we have recently been approved for a 1 year no cost extension. In the period of no-cost extension, we will repeat the long term experiments with repetitive subconcussive TBI (rTBI) , as well as repeat the histology for the rTBI and severe impact paradigms. Once we consider our results to be definitive, our intent is to publish this in the peer-review literature in the NCE period, and present at a national meeting, such as the Society for Neurocritical Care.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

As noted above, we feel that our results demonstrate potential clinical utility for administration of CN-105, both in the context of chronic prophylactic administration to mitigate the functional deficits and structural/histological sequelae associated with acute closed head injury and repetitive milder head injury. In the no-cost extension period, we will repeat these results, including the long term functional and behavioral assays and histological assessments associated with both models. Once we have replicated results, we will plan on presenting nationally and publishing within the NCE period.

As we have already completed Phase 1 (single and multiple escalating doses included as Appendix), and initiated a Phase 2 study in patients with intracranial hemorrhage, our intent will be to work with the DOD to establish the potential feasibility of designing an initial clinical first-in-disease state trial for patients with closed head injury.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

CN-105 was designed from the receptor binding face of apolipoprotein E, which is an endogenous protein that has been demonstrated to modify injury responses and outcomes after acute brain injury. In particular, we have demonstrated that CN-105 mimics the neuroprotective and anti-inflammatory actions of the native holoprotein. As there are no pharmacological interventions that have ever been demonstrated to improve functional outcomes after acute brain injury, this would represent the first-in class and first-in-disease-state therapeutic to improve outcomes following for acute brain injury. We are particularly excited by the fact that CN-105 improves long term functional outcomes and reduces chronic histological and structural changes associated with mild repetitive head injury.

What was the impact on other disciplines?

We have initiated a multi-center Phase 2 open label study evaluating the safety and feasibility of administering CN-105 in patients with spontaneous intracranial hemorrhage. Although this is a different patient population than soldiers with traumatic brain injury, many of the mechanisms of neuroinflammation, secondary neuronal injury, and development of cerebral edema are similar. In fact, parenchymal hemorrhage is a common pathological feature of brain injury associated with TBI. We feel that this study may facilitate an early clinical study in patients with traumatic brain injury.

What was the impact on technology transfer?

There is no new intellectual property that was developed during this reporting period.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report (we have initiated a phase 2 multicenter trial in spontaneous intracranial hemorrhage, as noted above)

- 5. CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report. We anticipate that we will fully utilize the budget in the no cost extension period, which will be consistent with eth costs necessary to complete all of the stated objectives of this proposal.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

There are no human subjects associated with any of the research activities included in this grant.

Significant changes in use or care of vertebrate animals

As described in the initial SOW, Institutional Animal Care and Use Committee approval and ACURO approval were obtained in the first month of this grant. All procedures described have remained compliant with these approvals.

Significant changes in use of biohazards and/or select agents

Nothing to report. There were no biohazards associated with this proposal.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

- **Publications, conference papers, and presentations**
Report only the major publication(s) resulting from the work under this award.

Journal publications.

We anticipate that submitting a peer review publication describing the effects of CN-105 when used as a prophylactic neuroprotectant therapy in our model of moderate to severe TBI and repetitive mild TBI in the coming year (NCE extension period), and also presentation in a national meeting.

Included as an Appendix are two manuscripts directly relevant to this award. The first is pursuant to the suggestion that we define the neuroprotective efficacy of CN-105 when administered after injury (this award is cited); the second demonstrates safety pharmacokinetic profile of CN-105 in a phase 1 study, which is important to translation to a clinical study (this work was not performed within the scope of the current award, which is not cited).

Laskowitz DT, Wang H, Chen T, Lubkin DT, Cantillana V, Tian-Ming T, Kernagis D, Zhou G, Macy G, Kolls BJ, Dawson HN” Neuroprotective pentapeptide CN-105 is associated with reduced sterile inflammation and improved functional outcome in a traumatic brain injury murine model” Scientific Reports, 2017:46461PMID: 28429734 (*this award was cited*)

Guptill JT, Raja S, Ramey S, Boakye-Agyeman F, Noveck R, Tu T-M, **Laskowitz DT**. Phase I, randomized, double-blind, placebo controlled study to determine the safety, tolerability, and pharmacokinetics of a single escalating dose and repeated doses of CN-105 in healthy adult subjects. J Clinical Pharmacology, 2016. PMID: 27990643

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

There are several other recent publications that are directly relevant to this proposal, with regard to the efficacy of CN-105 in preclinical models of acute brain injury:

Tu TM, Wang H, Dawson H, Kolls B, **Laskowitz DT**. "Apolipoprotein E mimetic peptide, CN-105, improves outcomes in ischemic stroke" Annals of Clinical and Translational Neurology 4(4):246-265, 2017 PMID: 28382306

Lei B, James ML, Liu J, Zhou G, Venkatramen TN, Lascola CD, Acheson S, Dubois LG, **Laskowitz DT**, Wang H Neuroprotective pentapeptide CN-105 improves functional and histological outcomes in a murine model of intracerebral hemorrhage Scientific Reports 6:34834, 2016 27713572

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

There was no new technologies or techniques.

- **Inventions, patent applications, and/or licenses**

There was no new IP filed during the course of this grant.

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

*Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5*

*Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.
Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Daniel Laskowitz, MD, MHS:
Project role:	PI
Nearest person month worked:	2.0
Contribution to project:	Dr. Laskowitz serves as the PI, and is responsible for study design and conduct, reporting. (unchanged)
Funding Support:	Cure Alzheimer's Foundation (0.12 calendar month) (described in next section)
Name:	Haichen Wang, MD
Project Role:	Co-investigator
Nearest person month worked:	4
Contribution to Project:	Responsible for animal surgical procedures; preclinical models of neurotrauma (unchanged)
Funding Support	Yunnan Valley Pharma contract to test new pharmacological therapies in stroke (3 calendar months)
Name:	Viviana Cantillina-Riquelme
Project Role:	Research analyst
Contribution to Project:	Animal behavior and functional testing, histology and unbiased formal stereology, data analysis
Nearest person month worked:	10
Funding support:	No other Funding support

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Since this proposal has initiated, the PI has had funded one additional grant, entitled “A novel apoE mimetic therapeutic peptide CN-105 attenuates AD pathology and improves functional outcomes in a murine model of Alzheimer’s disease”. In this current study, we will test the hypothesis that CN-105, delivered as a continuous infusion over 6 weeks, will reduce AD pathology and improve behavioural outcomes. There is no scientific or budgetary overlap. He contributes 0.12 calendar months/year towards this grant which runs from 2/1/17 through 1/31/2018.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Attached are two appendices; both are manuscripts that were published recently:

Laskowitz DT, Wang H, Chen T, Lubkin DT, Cantillana V, Tian-Ming T, Kernagis D, Zhou G, Macy G, Kolls BJ, Dawson HN” Neuroprotective pentapeptide CN-105 is associated with reduced sterile inflammation and improved functional outcome in a traumatic brain injury murine model” Scientific Reports, 2017:46461PMID: 28429734 (*this grant is cited*)

Guptill JT, Raja S, Ramey S, Boakye-Agyeman F, Noveck R, Tu T-M, **Laskowitz DT**. Phase I, randomized, double-blind, placebo controlled study to determine the safety, tolerability, and pharmacokinetics of a single escalating dose and repeated doses of CN-105 in healthy adult subjects. J Clinical Pharmacology, 2016. PMID: 27990643

SCIENTIFIC REPORTS

OPEN

Neuroprotective pentapeptide CN-105 is associated with reduced sterile inflammation and improved functional outcomes in a traumatic brain injury murine model

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Daniel T. Laskowitz^{1,2,3,*}, Haichen Wang^{1,*}, Tony Chen¹, David T. Lubkin¹, Viviana Cantillana¹, Tian Ming Tu^{1,4}, Dawn Kernagis^{1,†}, Guanen Zhou^{1,‡}, Gary Macy¹, Bradley J. Kolls¹ & Hana N. Dawson¹

At present, there are no proven pharmacological treatments demonstrated to improve long term functional outcomes following traumatic brain injury (TBI). In the setting of non-penetrating TBI, sterile brain inflammatory responses are associated with the development of cerebral edema, intracranial hypertension, and secondary neuronal injury. There is increasing evidence that endogenous apolipoprotein E (apoE) modifies the neuroinflammatory response through its role in downregulating glial activation, however, the intact apoE holoprotein does not cross the blood-brain barrier due to its size. To address this limitation, we developed a small 5 amino acid apoE mimetic peptide (CN-105) that mimics the polar face of the apoE helical domain involved in receptor interactions. The goal of this study was to investigate the therapeutic potential of CN-105 in a murine model of closed head injury. Treatment with CN-105 was associated with a durable improvement in functional outcomes as assessed by Rotarod and Morris Water Maze and a reduction in positive Fluoro-Jade B stained injured neurons and microglial activation. Administration of CN-105 was also associated with reduction in mRNA expression of a subset of inflammatory and immune-related genes.

Traumatic brain injury (TBI) remains a leading cause of mortality in the United States, accounting for approximately one third of injury-related deaths and with an annual prevalence of approximately 1.7 million patients^{1,2}. Long-term neurological morbidity following TBI is also common, and almost one half of all patients who are hospitalized with severe TBI will be left with significant neurocognitive sequelae that may impair quality of life^{3,4}. Unfortunately, therapeutic options for patients with TBI remain limited and are primarily focused on treating intracranial hypertension, optimizing cerebral perfusion, and providing supportive care.

One important mechanism that may contribute to progressive cerebral edema and delayed neuronal injury is the sterile neuroinflammatory response. Acute CNS injury triggers immune activation in the absence of microbial infection through the release of damage-associated molecular pattern molecules (DAMPs), which can have both adaptive and maladaptive effects on recovery⁵. Activation of microglia and recruitment of peripheral mononuclear cells into the CNS compartment represents the cornerstone of the CNS neuroinflammatory response; both are associated with the release of inflammatory cytokines. This inflammatory cascade contributes to oxidative stress, secondary neuronal injury, blood brain barrier break down, and resulting cerebral edema. Although a variety of immunomodulatory strategies, including the use of glucocorticoids⁶ have been tried in the setting of

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acute brain injury, there are no pharmacological interventions that have been associated with an improvement in long term functional outcomes⁷.

It is increasingly recognized that genetic influences play an important role in modifying acute brain injury responses and outcome after TBI. One of the most robust genetic associations with outcome after TBI is the apolipoprotein E (APOE - gene; apoE - protein) polymorphism. There are three distinct apoE protein isoforms, designated apoE2, apoE3, and apoE4. Although there are conflicting reports on the effect of APOE4 and poor prognosis after subarachnoid hemorrhage^{8,9} and intracranial hemorrhage^{10,11}, cumulative evidence suggests that APOE4 is associated with poor outcome after acute brain injury, and in particular following traumatic brain injury^{12–17}. These data underscore the importance that apoE plays in mediating CNS responses to acute injury and neurodegeneration.

The apoE protein was initially found to reduce glial activation and inflammatory cytokine release *in vitro*¹⁸, and these results were extended to our murine model of TBI, where the absence of endogenous apoE exacerbated post-injury neuroinflammation and development of cerebral edema¹⁹. However, the intact apoE holoprotein does not readily cross the blood brain barrier (BBB), and would thus be unsuitable for peripheral administration²⁰. To address this problem, we originally created a series of apoE mimetic peptides derived from the apoE receptor binding region (residues 130–150)^{21,22}. These peptides had robust efficacy in cell culture²² and improved functional and histological endpoints in preclinical models of CNS inflammation²³ and acute brain injury associated with many of the pathological features of clinical TBI, including intracranial²⁴ and subarachnoid hemorrhage^{25,26} and both closed skull and cortical contusion models of brain trauma^{27–30}.

In the current study, we developed a smaller peptide (CN-105) that was designed by modeling the polar receptor binding face of the helical apoE receptor binding region (Ac-VSRRR-amide). We now test the hypothesis that intravenous administration of CN-105 dampens neuroinflammatory responses and thus possibly improves functional outcomes in a murine model of closed head injury. Moreover, we address variables that may inform the translation of CN-105 to the clinical setting, including defining the optimal dosing paradigm and latency from injury in which treatment continues to exert neuroprotective effects. Since microglial activation and subsequent neuroinflammation via specific receptor interactions may be one mechanism by which apoE modifies outcome following CNS injury^{22,23,30–34}, we therefore also examined possible mechanisms of action by assessing histology and inflammatory gene expression.

Results

CN-105 is associated with improved behavioral function. The murine closed head injury model used in this study creates a diffuse injury which presents with vestibulomotor and cognitive dysfunction, neuroinflammation and a degenerating neuronal population the hippocampus and cortex^{19,35}. This model is designed to recapitulate the diffuse glial activation, cerebral edema, and injury to preferentially vulnerable areas of cortex and hippocampus that result in vestibulomotor dysfunction and persistent neurocognitive deficits associated with moderate clinical traumatic brain injury. To establish a dose response for CN-105 treatment following acute brain injury, we compared a range of dosing concentrations with vehicle following TBI. Two hours after injury, CN-105 was administered intravenously at doses of 0.0125, 0.05, 0.2, or 0.8 mg/kg or vehicle ($n = 11–12$ /group) and animals were subsequently tested for functional performance. We assessed vestibulomotor performance on the Rotarod, and compared the CN-105 treated mice to vehicle treated mice. When compared to vehicle, improved functional performance by Rotarod was demonstrated with the 0.05, 0.2 and 0.8 mg/kg doses ($p = 0.011$, 0.023 and 0.009, respectively as assessed by two level ANOVA) while the 0.0125 mg/kg dose did not show robust improvement ($p = 0.267$) (Fig. 1A). The Rotarod performance of sham injured controls is shown for illustrative comparison only and was not included in the statistical analysis (Fig. 1A). Thus, our dose-response results demonstrated that 0.05 mg/kg CN-105 was the lowest effective dose resulting in improved functional performance, and this dose was used in subsequent experiments.

Since neurocognitive impairment is a common aftermath of TBI in the clinical setting and in our model, mice receiving the 0.05 mg/kg dose also received long term functional assessment of spatial learning and memory at 28 days post-injury by quantifying Morris Water Maze latency³⁶. CN-105 treated mice significantly reduced time to escape to the platform compared to vehicle treatment ($p = 0.036$) (Fig. 1B), suggesting a reduction in cognitive impairment following injury. The Morris Water Maze performance of sham injured controls is shown for illustrative comparison only and was not included in the statistical analysis (Fig. 1B). Furthermore, the CN-105 treated group performed better on the probe trial (Fig. 1C) confirming the treatment better preserved memory ($p = 0.0079$) than treatment with vehicle. There was no difference in swim speed (Fig. 1D) suggesting motor differences were minimal between the two groups ($p = 0.4968$) at this time point.

After establishing dose response, we next determined if we could extend the therapeutic window of CN-105 administration following TBI in this murine model. The lowest effective dose (0.05 mg/kg) of CN-105 administered at 4 hours post-injury resulted in a significant and durable improvement in Rotarod latency as compared to vehicle (Fig. 1E). This dosing paradigm also conferred long-term improvement in neurocognitive performance as quantified by a reduced deficit on Morris Water Maze testing (Fig. 1F). Furthermore, the CN-105 treated mice performed better on the probe trial (Fig. 1G) confirming the treatment better preserved memory ($p = 0.0211$) than treatment with vehicle. There was no significant difference in swim speed (Fig. 1H) suggesting motor differences were minimal between the two groups at this time point ($p = 0.0567$). Pretreatment of mice 30 minutes prior to TBI had no additional effects on vestibulomotor and cognitive function (data not shown).

CN-105 is associated with reduced microgliosis and neuronal injury following TBI. Since microglial activation and subsequent neuroinflammation via specific receptor interactions may be one mechanism by which apoE modifies outcome following CNS injury^{22,23,30–34}, we assessed differences in microglial activation between 0.05 mg/kg CN-105 treated and vehicle treated animals 10 days post TBI. Although microgliosis is also

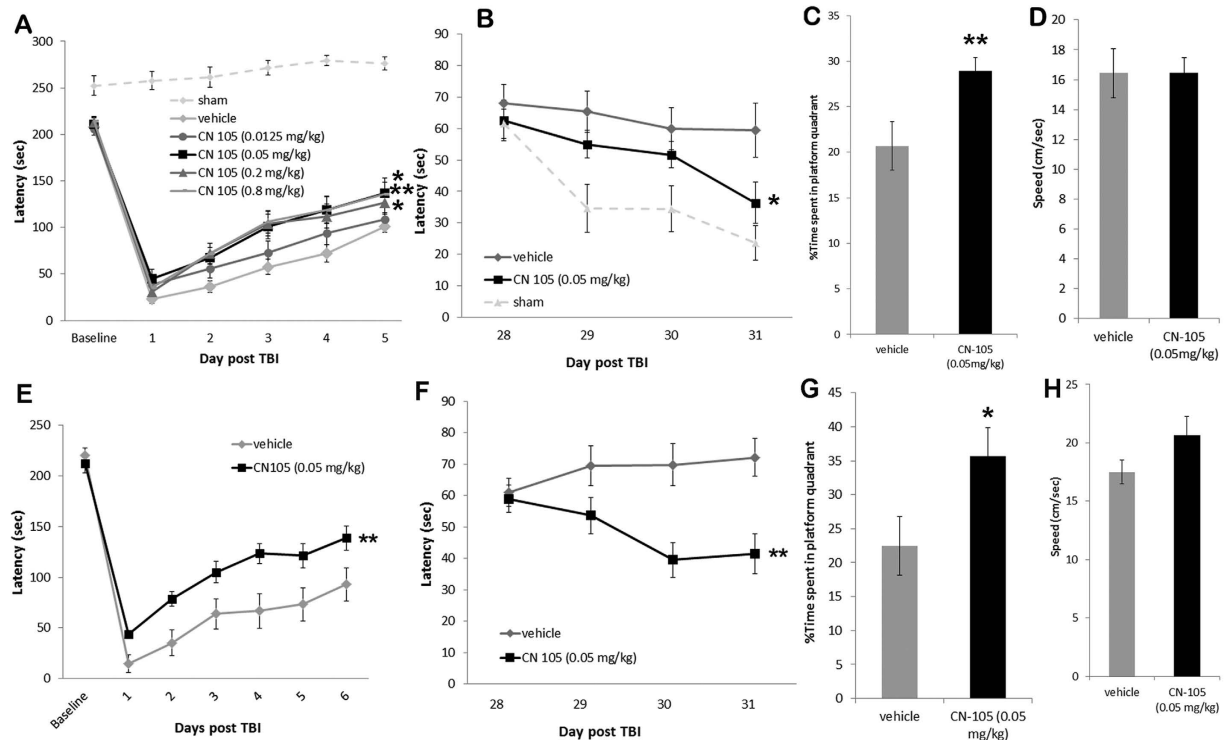


Figure 1. CN-105 improves vestibulomotor and cognitive function. Following TBI, intravenous administration of 0.05 mg/kg of CN-105 was the lowest effective dose resulting in improved vestibulomotor functional performance, as assessed by Rotarod (A) and cognitive function as assessed by the Morris Water Maze (B) and the MWM probe trial (C). There was no difference in swim speed on the MWM (D). CN-105 (0.05 mg/kg) administered 4 hours post-injury was also associated with a significant and durable improvement in Rotarod latency (E) and by reduced deficit on MWM (F) and the MWM probe trial (G). No significant difference in speed was detected between the two treatment groups (H). For comparison purposes results from sham mice are shown (dotted lines) in panels A and B but are not included in statistical calculations. Asterisks denote significant differences in performance as measured by ANOVA; * $p < 0.05$ and ** $p < 0.01$.

prominent in the cortex of closed head injury models, quantification of hippocampal inflammation is an important histological endpoint as it tends to be less variable than injury directly underlying the mechanical injury. A high level of microgliosis, as characterized by F4/80 immunostaining, is clearly evident in the hippocampus of the TBI injured mice (Fig. 2A), especially in the CA3 region (Fig. 2C), in the hilus of the dentate gyrus (Fig. 2E) and in the corpus callosum and fimbria surrounding the lateral ventricle (Fig. 2G). Treatment with CN-105 greatly reduced F4/80 immunopositive microglia in TBI injured mice as seen by microscopy (Fig. 2B,D,F and H). Quantification of F4/80 microglia in the dorsal hippocampus of TBI injured mice using unbiased stereology techniques showed a significant decrease in density of microglia in CN-105 treated mice compared to vehicle treated mice (Fig. 2I). Furthermore, microglia in the vehicle treated group displayed hypertrophy in the form of increased cytoplasmic volumes and thickness of processes indicative of microglial activation (Fig. 2C,E and G). Microglia from the CN-105 treated group (Fig. 2D,F and H) were almost exclusively small ramified microglia indicative of resting microglia with small cell bodies and thin, barely visible processes. Bushy, amoeboid, microglia that are associated with the phagocytic form were present throughout the hippocampus in the vehicle treated group only (Fig. 2E, arrowheads).

Degenerating neurons in the dentate gyrus have been previously reported and there is evidence that doublecortin-expressing late neural progenitors are relatively vulnerable to brain injury³⁷. Differences in neuronal injury between 0.05 mg/kg CN-105 treated and vehicle treated animals were assessed 24 hours post-injury by Fluoro-Jade B (FJB) staining. Significantly fewer Fluoro-Jade B positive degenerating neurons were present in the hilus of the dentate gyrus of CN-105 treated mice compared to vehicle treated mice (Fig. 2J and K). A number of vehicle treated mice also showed FJB staining in the CA3 region of the hippocampus, however, staining in the dentate gyrus is more consistent and was therefore used for quantification. Counting of the FJB positive neurons in every eighth brain slice of the dorsal hippocampus confirmed a significant decrease in the number of Fluoro-Jade B positive neurons ($p < 0.00001$) (Fig. 2L). Some of the FJB positive cells are located in the subgranular zone where adult neurogenesis occurs and may represent doublecortin (DCX)-expressing late neural progenitors. In sum, these results strongly suggest that administration of CN-105 is associated with a reduction of neuronal injury in the hippocampus.

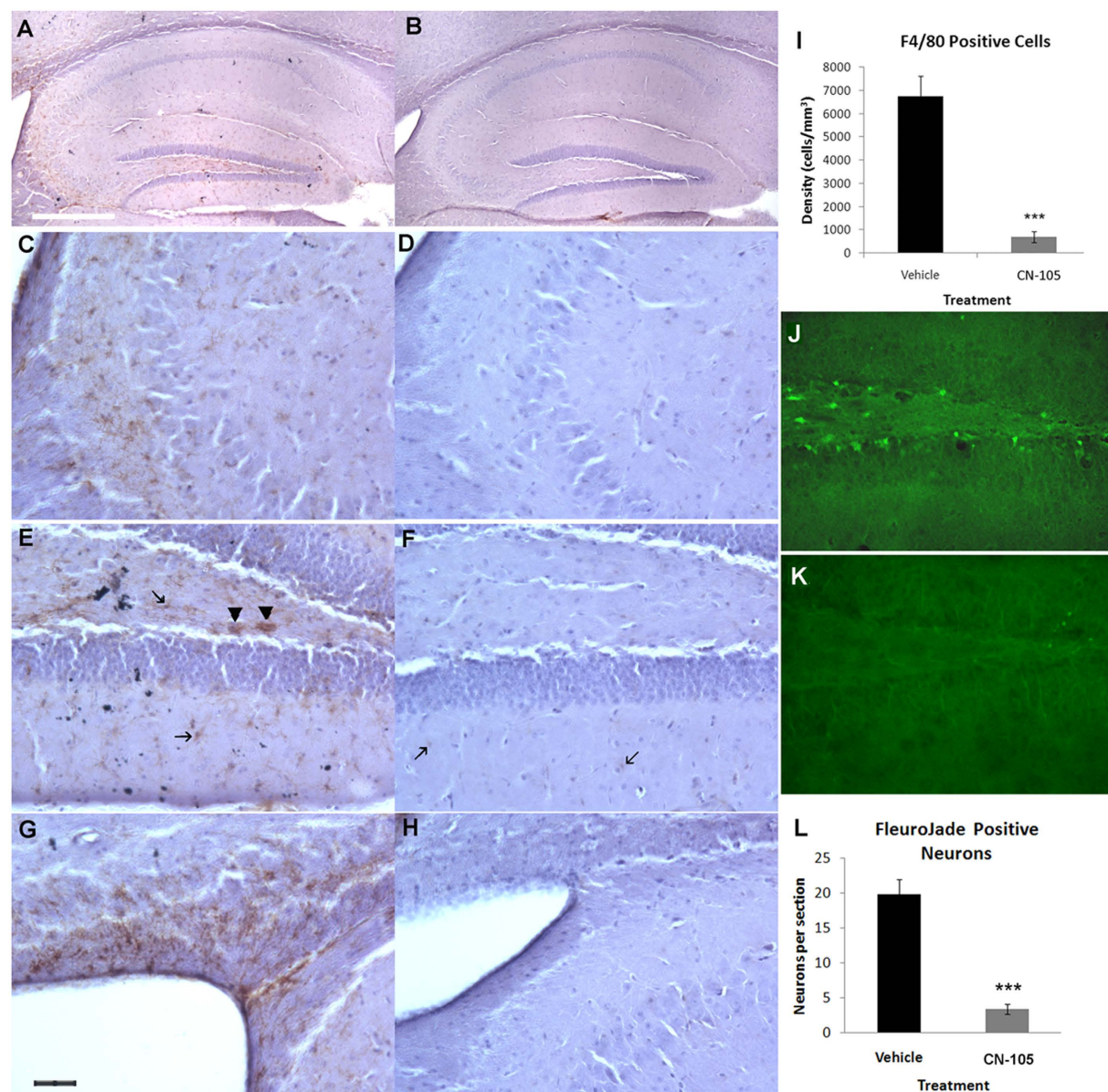


Figure 2. CN-105 reduces microgliosis and neuronal injury. Comparison images of activated F4/80 immunostained microglial in the hippocampus 10 days post-TBI, brain slices from vehicle (A,C,E,G) and CN-105 (B,D,F,H) treated mice, unbiased stereology confirms that treatment with CN-105 is associated with a reduction in microgliosis ($p = 0.0002$, ***) (I). Higher powered images of microglia in the CA3 region (C,D) in the polymorphic region (E,F) and in corpus callosum and fimbria of the periventricular region (G,H). Microglia are indicated in with arrows and phagocytic microglia with arrowheads in E and F. Images C-H are at 20x magnification and the black bar in G represents 40 μm . Images A and B are at 4x magnification and the white bar in A represents 250 μm . Comparison images of FJB-stained brain slices 24 hours post-TBI show degenerating neurons in the hilus of the dorsal dentate gyrus of TBI vehicle treated mice (J) which are significantly reduced by treatment with CN-105 (K), quantification $p < 0.0001$ (L).

CN-105 is associated with changes in inflammatory gene expression patterns following TBI. To further evaluate the effect of CN-105 on neuroinflammatory pathways, we assessed gene expression using a pathway array specific for inflammatory and immune responses. Four cohorts of mice were sacrificed at 24 hours post-injury; sham plus vehicle treated (S-v) mice, sham plus CN-105 treated (S-CN) mice, TBI plus vehicle treated (TBI-v) mice, and TBI plus CN-105 treated (TBI-CN) mice. CN-105 (0.05 mg/kg) and vehicle were administered 2 hours following injury. Relative gene expression based fold-change was calculated from raw threshold cycle data, using beta-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal standards for normalization. We first established the effect of TBI on these inflammatory pathways by normalizing RNA levels from injured animals receiving vehicle to non-injured sham animals receiving vehicle (Table 1). As expected, there were changes in mRNA levels of a number of inflammatory and immune-related genes in

Gene	S-v	S-CN-105	TBI-v	TBI-CN-105	S-CN-105/S-v	TBI-v/S-v	TBI-CN-105/S-v
Cxcl3	3.0E-05	2.3E-05	6.7E-01	1.6E+00	0.8	22171.7	54032.0
Ccl11	7.8E-05	2.5E-04	6.4E-01	1.8E-01	3.2	8199.7	2253.1
Ccr4	3.9E-06	6.0E-06	2.6E-02	3.1E-02	1.5	6537.5	7945.9
Ccl20	2.6E-05	1.1E-04	1.2E-01	7.9E-02	4.4	4574.0	3086.8
Tlr6	4.7E-04	6.2E-04	1.2E+00	7.0E-03	1.3	2528.8	14.9
Ccl24	3.2E-05	9.7E-05	7.6E-02	5.1E-03	3.0	2356.9	159.1
Myd88	7.8E-04	1.1E-03	1.8E+00	6.6E-03	1.5	2327.8	8.5
Cxcl9	6.8E-05	1.0E-04	1.5E-01	2.2E-03	1.5	2265.1	32.2
Lta	1.2E-04	2.6E-04	1.3E-01	1.1E-01	2.3	1133.5	917.0
Tnfsf14	2.5E-05	1.3E-04	2.5E-02	9.0E-03	5.2	1027.7	365.9
Il9	1.2E-05	*	1.2E-02	1.4E-02	**	957.7	1155.5
Ifng	7.3E-05	5.8E-05	5.6E-02	4.3E-03	0.8	767.1	59.7
Il10	1.9E-05	1.1E-04	1.5E-02	1.6E-03	5.7	766.1	82.2
Il1b	1.1E-03	2.0E-03	7.5E-01	4.6E-01	1.8	683.8	416.6
Il8rb	8.3E-06	6.1E-05	5.2E-03	5.4E-03	7.3	623.4	658.4
Il6	1.4E-04	1.1E-04	5.4E-02	1.8E-03	-1.3	395.1	13.2
Il1a	1.4E-03	1.9E-03	5.5E-01	5.4E-02	1.4	382.3	37.7
Cd40	5.4E-04	8.3E-04	1.6E-01	2.1E-02	1.5	301.4	38.8
Ccr2	2.6E-04	5.5E-04	7.1E-02	1.5E-03	2.1	271.6	5.6
Cxcl1	6.0E-05	1.6E-04	1.5E-02	1.8E-03	2.7	244.8	29.4
Ccr7	1.2E-04	2.1E-04	2.7E-02	3.4E-02	1.8	228.3	283.2
Il18rap	1.1E-04	4.5E-05	2.4E-02	6.6E-03	0.4	211.3	58.6
Tnf	2.8E-05	3.5E-05	5.5E-03	3.8E-02	1.3	197.3	1367.7
C4b	3.6E-03	7.0E-03	6.3E-01	5.9E-01	2.0	176.5	163.3
Il8ra	1.3E-05	*	2.2E-03	2.6E-03	**	168.5	196.8
Cxcr4	4.3E-03	6.6E-03	6.6E-01	9.0E-02	1.5	153.8	21.0
Cxcl11	2.4E-05	9.0E-05	3.4E-03	8.8E-03	3.8	141.3	372.3
Il6ra	3.4E-03	4.9E-03	4.8E-01	1.6E-01	1.5	140.3	47.9
Il22	4.3E-05	4.7E-04	5.6E-03	3.4E-03	10.8	129.4	77.8
Il1rn	2.3E-05	*	2.8E-03	1.7E-03	**	121.0	74.2
Ccl2	5.8E-04	8.3E-04	6.5E-02	2.5E-02	1.4	110.8	42.3
C3ar1	3.5E-03	4.1E-03	3.7E-01	1.2E-01	1.2	108.2	34.9
Il1f10	1.6E-05	*	1.5E-03	5.5E-03	**	93.4	352.3
Ccl4	3.7E-04	3.8E-04	3.0E-02	1.2E-02	1.0	83.4	32.3
Tlr4	3.1E-03	3.5E-03	1.8E-01	2.0E-02	1.1	59.2	6.5
Il23a	2.7E-05	8.0E-05	1.2E-03	2.3E-02	3.0	45.4	838.4
Ripk2	7.0E-03	1.1E-02	2.3E-01	2.1E-01	1.5	32.0	30.1
Tlr7	1.7E-03	2.6E-03	4.8E-02	9.1E-01	1.6	28.7	546.2
Cxcl10	9.2E-04	1.9E-03	2.5E-02	5.3E-04	2.1	27.6	-1.7
Tlr1	4.8E-04	1.0E-03	1.1E-02	7.4E-03	2.2	22.0	15.4
Tirap	3.8E-03	7.8E-03	7.9E-02	5.3E-02	2.1	21.0	14.0
Ltb	5.1E-04	1.0E-03	1.1E-02	3.9E-02	2.0	20.9	75.7
Ccl25	5.6E-03	1.4E-02	1.1E-01	9.5E-02	2.6	20.3	16.9
Hdac4	6.0E-02	9.1E-02	9.0E-01	8.3E-01	1.5	14.9	13.9
Itgb2	5.5E-03	1.3E-02	8.1E-02	5.3E-02	2.5	14.7	9.6
Ccr3	1.7E-03	2.1E-03	2.1E-02	1.1E-01	1.2	12.3	66.6
Ccl3	5.1E-04	8.0E-04	6.1E-03	8.9E-03	1.6	12.0	17.7
Il7	1.1E-04	3.2E-04	1.1E-03	5.9E-01	3.1	10.8	5569.8
Tlr2	9.1E-04	1.3E-03	9.0E-03	9.1E-03	1.4	9.9	10.0
Fasl	2.1E-04	1.9E-04	1.3E-03	6.7E-04	0.9	6.3	3.2
Ccl5	8.9E-04	1.1E-03	5.5E-03	3.4E-03	1.2	6.2	3.8
Cxcl2	6.6E-05	2.8E-04	3.5E-04	1.7E-03	4.2	5.3	25.5
Nfkb1	1.7E-02	3.1E-02	8.8E-02	5.3E-03	1.8	5.3	-3.1
Cebpb	6.8E-02	1.1E-01	3.5E-01	2.4E-01	1.6	5.2	3.5
Ccl7	1.3E-03	1.6E-03	4.4E-03	3.7E-03	1.3	3.5	2.9
C3	4.1E-04	7.0E-04	1.3E-03	1.9E-03	1.7	3.1	4.6
Continued							

Gene	S-v	S-CN-105	TBI-v	TBI-CN-105	S-CN-105/S-v	TBI-v/S-v	TBI-CN-105/S-v
Tlr3	1.5E-02	2.3E-02	4.5E-02	5.8E-02	1.6	3.0	3.9
Ccl22	1.2E-04	3.8E-04	3.6E-04	2.2E-02	3.1	2.9	176.2
Csf1	1.1E-02	1.9E-02	2.4E-02	1.2E-02	1.7	2.3	1.1
Fos	1.5E-01	2.3E-01	1.9E-01	1.7E-01	1.6	1.3	1.1
Tlr5	6.8E-04	6.3E-04	6.6E-04	1.7E-03	0.9	1.0	2.5
Il23r	1.1E-04	1.5E-04	1.4E-04	8.0E-04	1.4	-0.8	7.2
Tollip	1.6E-01	3.2E-01	1.5E-01	5.1E-02	1.9	-1.1	-3.2
Ccl19	1.9E-02	2.9E-02	1.7E-02	3.4E-03	1.5	-1.1	-5.8
Ccl17	2.6E-03	4.2E-03	1.7E-03	1.0E-03	1.6	-1.5	-2.6
Il10rb	1.5E-02	3.0E-02	8.4E-03	2.2E-02	2.0	-1.8	1.5
Flt3l	2.0E-03	3.0E-03	9.6E-04	7.1E-04	1.5	-2.1	-2.8
Ly96	3.8E-03	7.2E-03	1.2E-03	7.7E-02	1.9	-3.1	19.9
Il22ra2	2.4E-05	1.1E-04	7.5E-06	8.2E-02	4.8	-3.2	3448.3
Ccl8	7.5E-04	2.1E-03	1.3E-04	4.2E-03	2.8	-5.6	5.6
Ccr1	7.5E-04	7.1E-04	1.3E-04	3.0E-04	-1.1	-5.8	-2.5
Cxcl5	1.4E-03	2.5E-03	2.2E-04	1.4E+00	1.7	-6.5	957.4
Il1rap	3.1E-02	4.4E-02	1.8E-03	8.2E-04	1.5	-17.1	-37.5
Ccl12	3.6E-03	6.5E-03	2.1E-04	1.3E-02	1.8	-17.3	3.7
Il1r1	4.6E-03	1.2E-02	2.2E-04	7.5E-04	2.6	-21.5	-6.2
Nfatc3	8.7E-03	2.1E-02	3.2E-04	3.2E+00	2.4	-27.5	364.7
Il18	9.1E-02	1.8E-01	1.3E-03	9.6E-03	2.0	-68.6	-9.5
Bcl6	5.1E-02	6.6E-02	6.2E-04	5.7E-01	1.3	-82.1	11.2
Nr3c1	2.1E-01	4.0E-01	8.5E-04	1.2E-02	2.0	-243.3	-17.8
Ccl1	*	2.3E-05	6.3E-01	9.1E-01	**	**	**
Cd40lg	*	6.9E-05	4.9E-03	4.2E-03	**	**	**
Crp	*	*	5.9E-04	7.1E-04	**	**	**
Knlg1	*	1.4E-05	9.2E-03	8.5E-03	**	**	**
Nos2	8.8E-05	8.9E-05	*	1.6E-01	1.0	**	1779.4

Table 1. Inflammatory gene expression, ΔC_t values and fold change relative to Sham-vehicle.

Inflammatory gene expression 24 hours post sham or TBI injury. Data is organized by fold change of TBI-vehicle normalized to sham-vehicle. *Indicate mRNA expression was below the level of detection. **Indicate relative value could not be calculated because one of the components was below the level of detection.

response to the closed head injury. Of the 84 genes evaluated, 57 genes were upregulated, 12 genes were downregulated, and 10 genes were unchanged (Table 1).

Several of the most highly upregulated genes in this array 24 hours post TBI are members of the TLR signaling pathway, notably *Tlr6* and *Myd88*, and to a lesser extent *TLR4*, *TLR7*, *TLR1*, *Tirap*, *TLR2* and *Nfkb1*, the DNA binding unit of NF- κ B (Table 1). As expected the upregulation of the TLR pathway and activation of NF- κ B leads to the upregulation of a number of pro-inflammatory cytokine genes, including, *IL1 α* and β , *IL-6*, *IL-22*, and *TNF α* and *TNF β* . Also highly induced are the primary and secondary activators of macrophages, *INF γ* and the receptor for *CD40l*, *CD40*. Furthermore, chemokine genes such as *Cxcl3* also known as the growth-regulated protein (GRO), protein gamma and macrophage inflammatory protein-2-beta (MIP2b), *Ccl11* (eotaxin-1) and *Ccl20* (NIP3A), *Ccl24* (eotaxin-2), *Cxcl9* (MIG), *Cxcl1* and *Cxcl10* (Table 1) were also among the most upregulated genes following TBI. These chemokines control migration and adhesion of cells of the immune response such as monocytes, eosinophils, lymphocytes, and T-cells. Concurrently, the expression of the anti-inflammatory *IL-10* gene is highly increased after TBI. IL-10 expression is also activated through the TLR pathway. Interestingly IL-10 has been shown to play a role in autoimmune disease (reviewed in ref. 38). Of the 12 genes downregulated by TBI, *Nr3c1*, *Bcl6*, *IL18*, *Nfatc3* and *IL1r1* were some of most highly repressed genes. Of note is the robust downregulation of the *Bcl6* gene, which codes for a transcription repressor implicated in many cellular pathways to include inflammation.

After establishing the effect of TBI on inflammatory mRNA expression, we next assessed the effect of CN-105 following TBI (Tables 1 and 2; Fig. 3). Represented in Fig. 3 are genes from TBI-v brains that show a greater than 3 fold difference when compared to TBI-CN brains. Results of sham-v mice are also presented for comparison. The values for all 3 groups are shown relative to sham-v gene expression. Of the 57 genes that were upregulated by TBI, the overexpression of 28 genes was substantially (as defined by fold change >3) altered by treatment with CN-105 (Fig. 3A). Of note, the post-injury upregulation of TLR pathway genes such as *Tlr6*, *Myd88*, *TLR4*, and the DNA binding unit of NF- κ B post TBI was reduced by treatment, as were the TBI upregulated cytokines *IL6*, *IL1 α* , *IL10* and the primary activators of microglia/macrophages *INF γ* and *CD40* (Fig. 3A). Chemokine genes such as *Cxcl9*, *Cxcl10*, *Ccl24*, *Cxcl1* were also lower than in the vehicle treated TBI group, as were the genes coding for chemokine receptors *Ccr2* and *Cxcr4* and *IL18rap*, the accessory subunit of the heterodimeric receptor

Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
<i>Il22ra2</i>	10895	<i>Tlr3</i>	1.3	<i>Fasl</i>	−2.0
<i>Nfatc3</i>	10044	<i>Ccr7</i>	1.2	<i>Csf1</i>	−2.0
<i>Cxcl5</i>	6257	<i>Ccr4</i>	1.2	<i>Il1rap</i>	−2.2
<i>Bcl6</i>	921	<i>Il9</i>	1.2	<i>Ccl4</i>	−2.6
<i>Il7</i>	515	<i>Crp</i>	1.2	<i>Ccl2</i>	−2.6
<i>Ccl12</i>	64.2	<i>Il8ra</i>	1.2	<i>Tnfsf14</i>	−2.8
<i>Ly96</i>	61.2	<i>Il8rb</i>	1.1	<i>Tollip</i>	−2.9
<i>Ccl22</i>	60.2	<i>Tlr2</i>	1.0	<i>Il6ra</i>	−2.9
<i>Ccl8</i>	31.2	<i>Ripk2</i>	−1.1	<i>C3ar1</i>	−3.1
<i>Tlr7</i>	19.0	<i>Knq1</i>	−1.1	<i>Il18rap</i>	−3.6
<i>Il23a</i>	18.5	<i>Hdac4</i>	−1.1	<i>Ccl11</i>	−3.6
<i>Nr3c1</i>	13.7	<i>C4b</i>	−1.1	<i>Ccl19</i>	−5.2
<i>Il18</i>	7.2	<i>Fos</i>	−1.2	<i>Cxcr4</i>	−7.3
<i>Tnf</i>	6.9	<i>Cd40lg</i>	−1.2	<i>Cd40</i>	−7.8
<i>Il23r</i>	5.8	<i>Ccl7</i>	−1.2	<i>Cxcl1</i>	−8.3
<i>Ccr3</i>	5.4	<i>Ccl25</i>	−1.2	<i>Tlr4</i>	−9.0
<i>Cxcl2</i>	4.8	<i>Lta</i>	−1.2	<i>Il10</i>	−9.3
<i>Il1f10</i>	3.8	<i>Flt3l</i>	−1.4	<i>Il1a</i>	−10.1
<i>Ltb</i>	3.6	<i>Tlr1</i>	−1.4	<i>Ifng</i>	−12.9
<i>Il1r1</i>	3.5	<i>Ccl20</i>	−1.5	<i>Ccl24</i>	−14.8
<i>Cxcl11</i>	2.6	<i>Cebpb</i>	−1.5	<i>Nfkb1</i>	−16.6
<i>Il10rb</i>	2.6	<i>Tirap</i>	−1.5	<i>Il6</i>	−29.8
<i>Tlr5</i>	2.5	<i>Itgb2</i>	−1.5	<i>Cxcl10</i>	−47.9
<i>Cxcl3</i>	2.4	<i>Il1rn</i>	−1.6	<i>Ccr2</i>	−48.3
<i>Ccr1</i>	2.3	<i>Ccl5</i>	−1.6	<i>Cxcl9</i>	−70.3
<i>C3</i>	1.5	<i>Il1b</i>	−1.6	<i>Tlr6</i>	−170
<i>Ccl3</i>	1.5	<i>Il22</i>	−1.7	<i>Myd88</i>	−273
<i>Ccl1</i>	1.5	<i>Ccl17</i>	−1.7	<i>Nos2</i>	*

Table 2. Inflammatory gene expression of CN-105 relative to vehicle treated group following TBI. Fold Change-Inflammatory gene expression 24 hours post TBI with CN-105 treatment relative to vehicle. Greater than 3 fold change-light gray, unchanged-unshaded, below threshold of detection dark-gray. *Indicate mRNA expression was below the level of detection.

for IL18, required for the activation of NF- κ B and JNK in response to IL18. The expression of several genes upregulated by TBI is upregulated to a higher level by treatment with CN-105 (Fig. 3A). Several of these genes such as *Ccl22* and *Il-7* are associated with recovery and repair following injury. *Il-7* has been shown to promote neuronal survival, while *Ccl22* is associated with M2 macrophage polarization. Although the function of *IL1f10* (corresponds to *IL-38* in humans) is a recently discovered cytokine it has been shown to inhibit inflammatory responses^{39–41}. TBI-associated downregulation of the *Bcl6* was significantly reversed, to above sham levels by CN-105 (Fig. 3B). In aggregate, these results are consistent with the hypothesis that administration of CN-105, has the overall effect of downregulating neuroinflammatory responses following TBI.

To assess the effects of CN-105 on inflammatory gene expression on control mice, mRNA from sham CN-105 treated mice was compared to sham vehicle treated mice. Gene expression increase greater than 3 fold was observed in 13 genes (Table 1). No downregulation of gene expression was observed.

CN-105 demonstrates penetration into the CNS compartment. One rationale for creating the smaller apoE mimetic peptides was to enhance CNS penetration and possibility of noninvasive mechanisms of delivery (for example, intranasal administration). Of note, a higher dose of CN-105 and different mouse strain was utilized in these outsourced experiments (CD-1 vs. C57-BL/6), although prior reports suggest that this would not affect pharmacokinetic results^{42,43}. To define pharmacokinetic parameters, we performed pharmacokinetic studies of CN-105 following a single 1.92 mg/kg (100 μ Ci/kg) intravenous injection of stable, [¹⁴C]-radiolabeled CN-105 peptide (Fig. 4). Our analysis demonstrated an initial (0–4 hours post injection) plasma half-life of 29 minutes, with t_{max} in serum of 5 minutes after injection (the first time point tested). The t_{max} in the brain was 30 minutes after injection. The total CNS exposure (0–4 hours post injection) as calculated by area under the tissue concentration curve divided by area under the plasma concentration curve was 60% (Fig. 4). The progressive increase in radioactivity in the brain as compared to blood (3.6% at 5 minutes and 170% at 24 hours) demonstrates access to the CNS compartment rather than the blood in the cerebral microvasculature (Table 3). Of note, these studies were performed in uninjured animals, and it is likely that levels would be higher following forms of CNS injury that are associated with breach of the blood brain barrier.

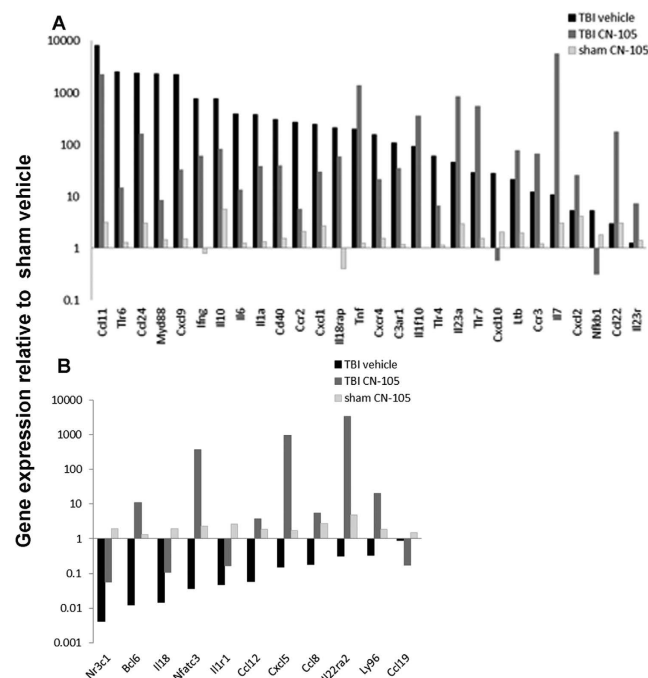


Figure 3. CN-105 ameliorates changes in inflammatory gene expression. One day post-injury, differential inflammatory gene expression analysis demonstrated changes in gene expression relative to sham vehicle treated controls for TBI CN-105 treated and untreated mice. Results are organized by upregulated (**A**) and downregulated (**B**) expression of genes from vehicle treated TBI mice relative to vehicle treated sham mice. Results from CN-105 treated TBI and sham mice relative to vehicle treated sham mice are also included. Only genes from TBI CN-105 treated mice that showed more than a 3 fold change when compared to TBI vehicle treated mice are presented.

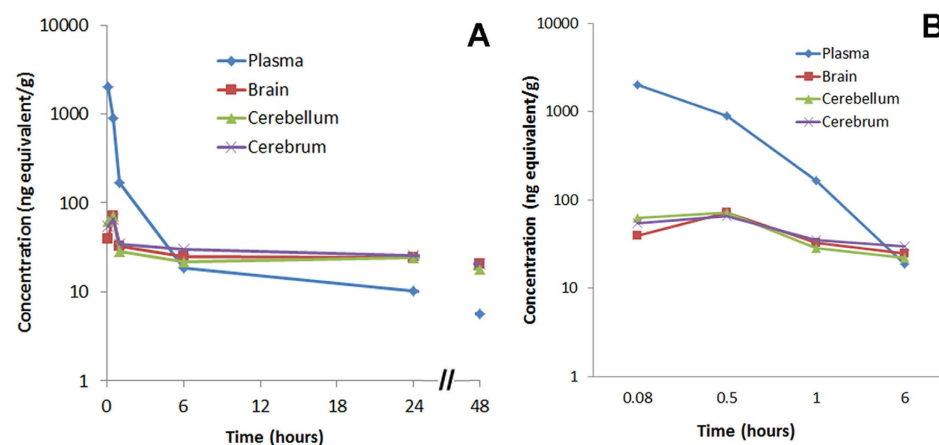


Figure 4. Pharmacokinetic studies of CN-105 demonstrate CNS bioavailability. Concentration of [^{14}C]-radioactivity in the plasma and central nervous system of male CD-1 mice following an intravenous dose of [^{14}C]-radiolabeled CN-105 peptide (**A**). For clarity, the concentration of [^{14}C]-radioactivity during the first 6 hours only is depicted in (**B**).

	Radioactivity (ng equiv/g) in tissue following bolus (hours = h)					
	0.08 h	0.5 h	1 h	6 h	24 h	48 h
Blood (Cardiac)	1102	500	95	15	14.1	15.5
Brain	39.5	71.2	32.5	24.9	24.1	20.9
% Brain/Blood	3.58%	14%	34%	167%	170%	135%

Table 3. Percent of radioactivity contributed to blood in the brain microvasculature.

Discussion

In the current study we demonstrate that a rationally designed, five amino acid peptide that mimics the polar face of the receptor binding region of endogenous apoE (CN-105) improves microscopic and functional endpoints following acute traumatic brain injury. In particular, treatment with CN-105 was associated with a reduction in neuroinflammatory responses, as assessed by histological evidence of reduced microgliosis and downregulated expression of the majority of inflammatory gene mRNAs following injury. These findings were accompanied by a reduction in neuronal injury in the hippocampus, which is selectively vulnerable following trauma⁴⁴. Treatment with CN-105 after injury was well tolerated and associated with durable functional benefits, as defined by a reduction in vestibulomotor and neurocognitive deficits. Our results are consistent with prior data demonstrating that apoE and apoE-mimetic peptides decrease neuroinflammatory responses and secondary cell death in cell culture and animal models of acute brain injury^{22,23,45–48}.

The activity of anti-inflammatory drugs may be directly assessed through changes in the levels of pro- and anti-inflammatory mediators as well as the reduction of the number and activation state of inflammatory cells. However, cytokine mediators may work in very low concentrations, which may contribute to conflicting reports regarding level and time of activation after injury. For example, although several groups have reported that mRNA levels of inflammatory cytokines and chemokines return to pre-injury levels by 24 hours^{49–51}, other groups report that increased levels persist for 24 hours or longer in rodents and human patients, reviewed in refs 52 and 53.

To date, a body of microglial research has been published using M1 and M2 polarization phenotypes based on macrophage terminology with the assumption that microglia mirror the function of peripheral macrophage in the brain. It is often difficult to characterize endogenous microglia (primitive macrophage entering the embryonic brain and maintained in the brain by cell division) from hematogenous macrophage that are recruited into the brain following acute injury. However, the fact that the number and activation status (determined by morphological analysis) of microglia following injury is also decreased by treatment with CN-105 is consistent with the anti-inflammatory actions of this peptide.

Here we report that many inflammatory indicators are still greatly elevated in our TBI model at 24 hours post injury and while the upregulation of several traditional indicators of inflammation are decreased by treatment with CN-105, some such as TNF α remain elevated. It is important to note that mRNA levels cannot be used as surrogates for corresponding protein levels without verification, especially in the setting of acute brain injury. Although the clues provided as to the mechanism of TBI inflammation and the downregulation by CN-105 is exciting, this mechanistic data needs to be regarded as preliminary, and results need to be confirmed by protein analysis. However, recent published reports have indicated that some cytokines that have initially been thought to be detrimental in propagating the immune response, such as TNF- α , may actually have a dichotomous effect, exacerbating inflammation in the initial stages while aiding in recovery in the later stages^{54–56} and reviewed in refs 57 and 58. Furthermore, there is evidence that several of the genes that show increased expression after injury have also demonstrated increased expression in recent studies. For example, MyD88, an adaptor protein for inflammatory pathways that include signal transduction via the TLR and IL-1 receptor families, was significantly increased after experimental TBI in rodent models and in the human brain after TBI^{59,60}. Also, Koedel and colleagues demonstrated that loss of MyD88 reduced acute brain injury and improved neurological status⁶¹. It is promising that our experiments show that our model of TBI increases many of these indicators of inflammation, which are reduced by treatment with CN-105.

The mechanism(s) by which apoE and CN-105 are able to modulate the inflammatory response of the brain to injury remain incompletely defined. One potential mechanism by which apoE and apoE mimetic peptides may affect brain injury responses is via specific receptor interactions that initiate a signaling cascade that modify downstream expression of inflammatory genes. It has been shown that glial secretion of apoE is upregulated in the injured brain, and although there is likely redundancy between a number of apoE receptors, the LRP-1 receptor appears to play a primary role in mediating its anti-inflammatory effect in an isoform-specific fashion^{62,63}. Moreover, apoE and apoE peptides have been demonstrated to bind the LRP receptor^{32,64}, and interaction with the LRP receptor initiates a signaling cascade associated with the downregulation of inflammatory phenotype, an effect that is not observed in LRP-deficient microglia^{7,33,34}. Moreover, apoE and apoE mimetic peptides directly reduced excitotoxic neuronal injury, an effect that is likely mediated by indirect effects of apoE receptors on the NMDA receptor complex via the cytoplasmic adaptor protein PSD-95^{31,45,48}. ApoE-LRP interactions have also been associated with a decrease in the translocation of the transcription factor NF- κ B which would also have the net effect of inhibiting inflammation following injury⁶⁵. This mechanism is consistent with the differential gene expression results in the current study, which demonstrated a decrease in Nfkb1 gene expression and the expression of downstream inflammatory cytokines 24 hours post-injury. The reduction in cytokines could also result from the finding that CN-105 suppresses the upregulation of the gene for the DNA binding p50 unit of NF- κ B (*Nfkb1*) (Fig. 3) that is seen 24 hours post TBI (Fig. 5) which would be expected to further reduce the inflammatory response mediated by NF- κ B activation.

Our gene expression data suggest that the early changes in NF- κ B, chemokines and cytokines may be linked to changes in BCL6 and other factors that are key in regulating the TLR mediated sterile inflammation pathway. The possibility that the apoE mimetic peptide modifies sterile inflammation in the central nervous system is particularly relevant after traumatic brain injury, where glial activation and neuroinflammatory responses exacerbate secondary tissue injury. Toll like receptors (TLRs) have been found to have a role in models of sterile inflammation (reviewed in refs 66 and 67)^{67–69}. TLR4 and TLR6 complexed with the scavenger receptor CD36 has been shown to signal through the intracellular adapter, MyD88, and NF- κ B to stimulate chemokine (*Cxcl1* and *Cxcl2*) and cytokine (*IL-1 α* , *IL-6*, *IL-10*, *Inf- γ* and *TNF- α*) expression⁷⁰. Here we report that twenty-four hours after TBI, many genes in this signaling pathway are upregulated (Fig. 5A and B, Table 1) with *Myd88* and *TLR6* showing the highest level of upregulation, and TLR4, IL-1 α , IL-6, IL-10 and *Cxcl1* demonstrating a moderate level of upregulation. In addition, it has been recently discovered that the leukemia oncogene Bcl6 is the master regulator

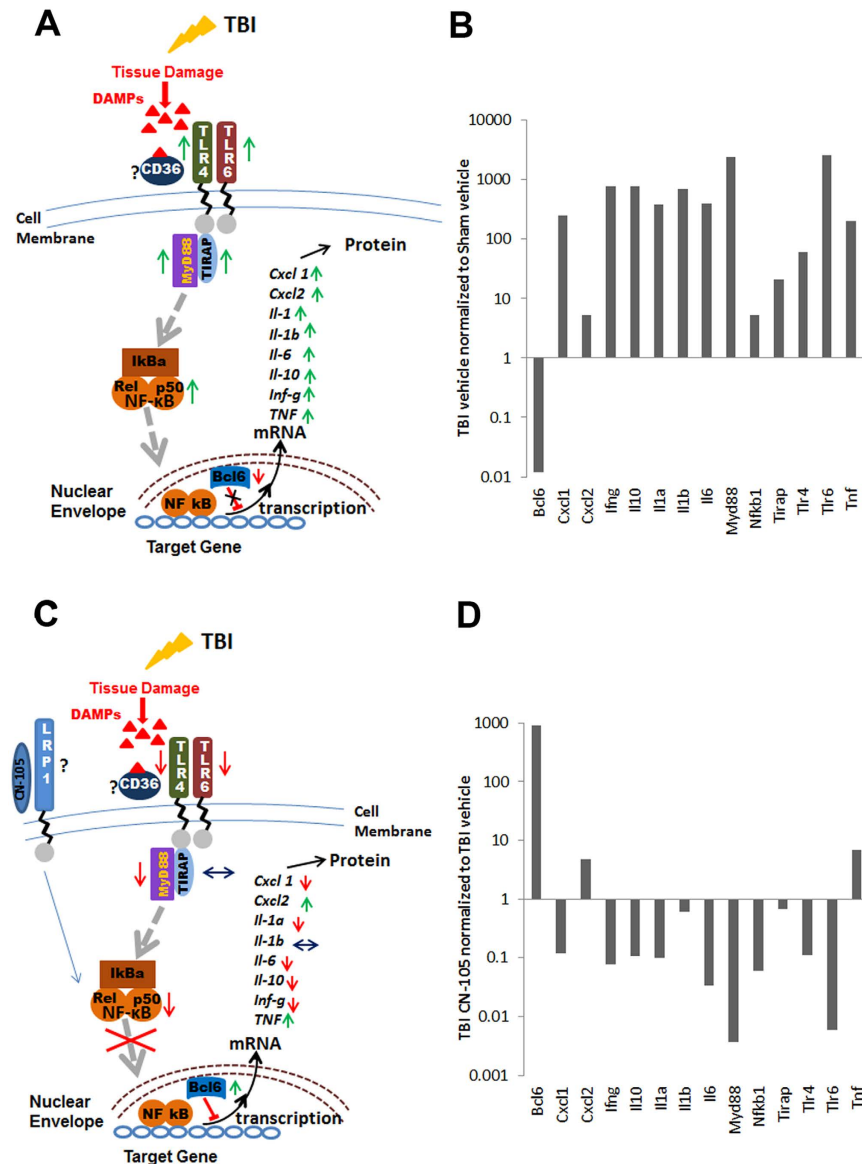


Figure 5. TLR signaling is downregulated by CN-105. (A) A schematic of the signaling pathway proposed for sterile inflammation post injury. Green arrows indicate upregulation and red arrows downregulation of genes 24 hours after TBI. (B) Graphic representation of genes 24 hours post TBI demonstrating fold change compared to sham control. (C) CN-105 modifies the post-injury inflammatory response. Arrows indicate reduced upregulation (green) and reduced downregulation (red) of gene expression when TBI injured mice are treated with CN-105 as compared to vehicle. (D) Graphic representation of differential gene expression 24 hours post TBI demonstrating fold change in gene expression of CN-105 treated mice normalized to vehicle treated TBI mice.

of many crucial pathways including NF-KB signaling related inflammation^{71–73}. As predicted by the published data, the repression of the gene for Bcl6 by TBI in our model coincides with increased levels of transcription of several TLR genes, *Myd88*, *CD40*, *INF γ* , *IL-1a*, *IL-10*, *IL-6* (Fig. 5A and B) along with the upregulation of *BCL6* mRNA the majority of those same genes is repressed in TBI mice treated with CN-105 (Fig. 5C and D). Thus, it is plausible that CN-105 modifies the acute post-injury neuroinflammatory response by interaction with LRP1, resulting in reduction of inflammatory genes mediated by the NF-KB and BCL-6 regulated pathways and thus ultimately reducing TLR mediated sterile inflammation and mitigating secondary neuronal injury, resulting in durable functional benefit. It is also possible that CN-105 has a direct effect on blood brain barrier integrity. This is consistent with recent results demonstrating that CN-105 reduced cerebral edema in a murine model of intracranial hemorrhage⁷⁴, and that apolipoprotein E may directly effect the blood brain barrier through regulation of tight junctions^{63,75}.

There are a number of challenges associated with the translation of a new therapeutic strategy to the clinical setting. One advantage of a therapeutic strategy that modifies post-traumatic neuroinflammatory responses is the

potential for a relatively long therapeutic window, as glial activation and the development of cerebral edema peaks during the first several days of injury. In this regard, defining the post-injury latency during which a therapeutic intervention retains its beneficial effect is an important variable in informing a pilot clinical trial. In the current trial, we found that pre-treatment with CN-105 was not necessary. In fact, functional improvement was retained when peptide was administered up to 4 hours following injury. In further studies, this temporal window should be further extended.

Several limitations to the current study should be addressed. For example, apoE-based peptides derived from the receptor binding region of apoE are not an ideal model to study the isoform specific effects of the endogenous holoprotein, as the amino acid substitutions that define the common APOE polymorphisms flank the receptor binding region, and likely modulate receptor binding via allosteric effects⁷⁶. Another potential limitation in these results is that, although the differential gene expression results are consistent with the histological features of reduced microgliosis and CNS inflammation as a function of CN-105 administered post-injury, it should also be noted that this conclusion may be biased by the fact that the differential gene assay was focused on expression of inflammatory mediators.

Given the failure of multiple neuroprotective trials, the translatability of rodent models to clinical TBI remains an area of debate. For example, there are a number of limitations inherent to all rodent models of traumatic brain injury. This includes the lissencephalic nature of rodent brains, which precludes testing of higher and more subtle cortical functions that are directly applicable to the clinical setting. Moreover, rodent brains have reduced ratio of white: grey matter as compared to primates, and thus are not ideal to model the diffuse axonal injury that may occur in the clinical setting. Murine TBI models also largely fail to recapitulate the biomechanical forces transmitted to human brains. Despite these limitations, a number of murine models have been developed to model focal injury (controlled cortical impact models) and closed head injury. It is important to note that all of these models have potential limitations and advantages, and may provide complementary information (reviewed in refs 77 and 78). Our model of closed head injury was chosen because it replicates many of the histological features (diffuse gliosis, hippocampal injury) and clinical sequelae (vestibulomotor deficits and long term neurocognitive deficits) associated with human moderate closed head injury. As noted above, although all rodent TBI models have relative advantages and liabilities, we have recently demonstrated that CN-105 reduces cerebral edema and improves functional outcome in a model of intracranial hemorrhage⁷⁴. This supports recent observations from our laboratory and others that apoE based peptides improve other facets of TBI pathology, including cerebral ischemia⁷⁹, intraparenchymal hemorrhage⁷⁴, and subarachnoid hemorrhage^{25,26}. An additional consideration is that, although an overly robust neuroinflammatory response may exacerbate tissue injury in the acute setting, there is evidence that activated glia may play an adaptive role in the subacute setting by providing trophic support and mediating adaptive synaptic reorganization^{80,81}. Finally, although peptide-based therapeutics may be appropriate for acute brain injury, they are likely to have limited oral bioavailability, and small molecule mimetics would likely be more appropriate for chronic neurodegenerative conditions associated with CNS inflammation.

In conclusion, we find that treatment with a small five amino acid residue peptide derived from the receptor binding region of apoE improved functional outcomes following closed head injury. These functional improvements in vestibulomotor and neurocognitive behavior were durable throughout the 31 day testing period, and were retained even when the first administration of apoE peptide was withheld until 4 hours following TBI. Moreover, administration of CN-105 peptide was well tolerated, and these functional improvements were associated with a reduction in hippocampal neuronal injury, microgliosis, and inflammatory gene expression. The further development of apoE based therapeutics represents a promising therapeutic strategy in the treatment of acute brain injury.

Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Duke University Institutional Animal Care and Use Committee, Durham North Carolina, protocol #: A030-12-02. All surgery was performed under isoflurane anesthesia.

Closed Head Injury Model. The murine closed head injury model used in this study was previously described²⁹. The closed head impact results in injury to selectively vulnerable neurons in cortex and hippocampus, and is associated with vestibulomotor deficits and long term neurocognitive deficits. Although animals do lose body weight, they rapidly regain spontaneous ventilation, righting reflex, and the ability to ambulate. Briefly, 12–14 week-old C57Bl/6J male mice (Jackson Laboratories, Bar Harbor, ME) were used. The trachea was intubated after anesthesia induction with 4.6% isoflurane and the lungs were mechanically ventilated with 1.6% isoflurane in 30% O₂/70% N₂. Core body temperature was maintained at 37 °C through a rectal probe. To avoid basilar skull fracture, ear bars are not used. The animal was secured in a stereotactic device in a prone position on a molded acrylic cast with surgical tape across the shoulders. The intubation tube was also secured to the acrylic cast with tape. The acrylic cast is designed to allow the mouse to have 3 mm of space below the head to allow for acceleration/deceleration of the head in this position. The head was shaved and the scalp was incised to expose the skull and identify anatomical landmarks. A concave 3-mm metallic disc was adhered to the skull immediately caudal to bregma. A 2.0-mm diameter pneumatic impactor (Air-Power Inc., High Point, NC) was used to deliver a single midline impact to the center of the disc surface. The impactor was discharged at 6.8 ± 0.2 m/second with a head displacement of 3 mm. After impact, the animals were allowed to recover spontaneous ventilation and then the tracheas were extubated. Mice were allowed free access to food and water. Sham mice were treated identically except for the absence of impact. All mice were housed in the same facility.

Drug Administration. CN-105 (Ac-VSRRR-amide) was synthesized by Polypeptide Inc. (San Diego, CA) to a purity of >99%, and was delivered in sterile normal saline. Animals were placed in a restrainer (Harvard Apparatus, Holliston, MA), and a single intravenous dose of drug (0.05 mg/kg) was administered by tail vein in a volume of 100 μ L. Vehicle treated animals received intravenous injection of 100 μ L of normal saline at the same time points. Animals were assigned to treatment group by a coded study identification number after injury using a paper randomization protocol. A block randomization scheme was used, so that an equal number of animals were randomized to each of the treatment groups during concurrent experiments.

Immunohistochemistry. To assess the effects of CN-105 on inflammation, neuronal injury and neuronal loss, immunohistochemical (IHC) staining was performed using the F4/80 antibody (a marker for mature microglia and macrophages; rat monoclonal, 1:10,000; Serotec, Raleigh, NC) and the Fluoro-Jade B stain (a marker of degenerating neurons; Histo-Chem Inc. Jefferson, AR) on days 10 and 1, respectively, after TBI. IHC was performed on separate cohorts of mice from those used in neurobehavioral tests. As previously described⁸², mice were anesthetized, euthanized, and perfused with 30 ml phosphate-buffered saline (PBS) via transcardiac puncture. The brains were then immersed in buffered formalin overnight and saturated with 30% sucrose in buffer. Sagittal sections (40 μ m) were sliced on a freezing microtome and collected in cryoprotectant solution. For histological assessment the following were used: Secondary antibody, biotinylated goat anti-mouse IgG (1:3,000), ABC, and DAB all from Vector Laboratories, Inc., Burlingame, CA. and Gill's Hematoxylin, Fisher Scientific, Fair Lawn, NJ.

Cell Quantification and Image Analysis. Prior to quantification, all slides were coded and the analyst was blinded to avoid experimenter bias. For the F4/80 quantification the brains of 5 TBI treated and 6 TBI vehicle treated mice (4–6 sections/mouse) were counted. Every eighth section of the dorsal hippocampus, according to the Paxinos and Franklin (2001) mouse brain atlas, was analyzed using the Stereo Investigator 7.0 software (MicroBrightField, Williston, VT). The entire hippocampus was outlined using a 4x objective. Immunopositive microglia with visible nuclei were identified with a 20x objective, and the total numbers were estimated with the optical fractionator method (West *et al.*, 1991). An average of 12 counting frames was analyzed per section, the grid size was 450 \times 450 μ m, and the disector dimensions were 80 \times 80 \times 10 μ m. As we did not quantify through the entire hippocampus, results are presented as objects per unit volume (mm³) to allow comparability between animals (number of objects estimated/total volume evaluated). Group averages for vehicle and CN-105 treated animals were generated and compared using the Student's *t* test function (GraphPad Prism software).

For Fluoro-Jade B, brain sections from 12 TBI mice (6 vehicle and 6 CN-105) containing the dorsal hippocampus (5–6 sections per animal) were examined for degenerating neurons using an epifluorescence microscope (Nikon, Tokyo, Japan) with a medium band blue excitation (Nikon B-2A, 450–490 nm) filter set. Images of the dentate gyrus were acquired and a virtual grid was placed over the image. Degenerating neurons were counted and the total number of Fluoro-Jade B-positive neurons per every eighth brain slice was recorded. Group averages for vehicle and CN-105 treated animals were generated and compared using the Student's *t* test function (GraphPad Prism software).

Testing of Functional Deficits. Mice were randomly assigned to treatment groups immediately following injury and all behavioral evaluations were performed by investigators blinded to treatment.

An automated Rotarod (Ugo Basile, Comerio, Italy) was used to assess vestibulomotor function⁸³. On the day prior to injury, mice (*n* = 11–12 mice per group) underwent one training trial at an accelerating rotational speed (4–40 rpm) for at least 200 seconds and then three additional test trials with the same accelerating rotational speed. The average time to fall from the rotating cylinder in the test trials was recorded as baseline latency. Mice were tested on consecutive days post-injury and received three consecutive daily trials with accelerating rotational speed (inter-trial interval = 15 minutes) (*n* = 10–12 mice). The average latency to fall from the rod was recorded. Mice unable to grasp the rotating rod were given a latency value of 0 seconds.

As described previously the Morris Water Maze assesses spatial learning and memory by testing the ability of mice to locate a submerged platform³⁶. The mice were placed in a pool (105 cm diameter) filled with room temperature (25 °C) water made opaque with fat free powdered milk and allowed up to 90 seconds to locate the submerged platform. The mice performed four trials/day for 4 consecutive days (inter-trial interval = 30 min). The mice were introduced in varying quadrants of the pool for each trial but the location of the platform never varied. The latency to locate the platform was recorded, and the 4 trials per day were averaged. Mice were tested on days 28–31 post-injury (*n* = 11–12 mice per group). A probe trial was administered on day 4 of the experiment 3 hours after the last test trial was completed. For the probe trial, the platform was removed and the mice were allowed to swim freely for 60 seconds. The percent of the time the mice spent in the platform quadrant was quantified.

To ensure that vision was intact in TBI injured mice a separate cohort of 6 TBI and 6 sham mice was assessed. On day 28 a visible platform with a flag attached to a wire that extended above the platform was placed in the pool and mice were introduced in varying quadrants of the pool for 4 trials, the latency was recorded. An hour following the visible trial the flag was removed from the platform and the platform was placed in a new location and four new trials were performed and the latency was recorded. The four trials were averaged.

RNA extraction and RT-PCR. Frozen, pulverized whole brain tissue was processed for RNA extraction from a separate cohort of treated and untreated injured animals on days 1 post-injury (CN-105, *n* = 4; vehicle, *n* = 3; Control (sham-operated), *n* = 3). Total RNA was extracted from frozen, pulverized whole brain tissue using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). RNA quantity and quality was assessed with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and by agarose gel electrophoresis. Only samples with a 260/280 ratio between 1.9–2.1, and a 260/230 ratio greater than 2.0, were

further processed. First strand complementary DNA (cDNA) was generated from 2 µg total RNA using the RT² First strand kit (SABiosciences, Frederick, MD.)

Gene expression was measured using the Mouse Inflammatory Response and Autoimmunity PCR Array (SABiosciences, Frederick, MD), which profiles the expression of 84 genes related to inflammatory and autoimmune processes. RT-PCR was performed according to manufacturer's instructions. Quality of the cDNA and PCR efficiency was verified by housekeeping genes and RT-PCR controls included in the PCR Array.

Gene expression data analysis. Raw RT-PCR data were analyzed using the Web-Based PCR Array Data Analysis software (SABiosciences). ΔC_t values and $\Delta\Delta C_t$ - based fold-change were calculated from raw threshold cycle data, using beta-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal standards for normalization. Fold changes were then normalized against sham-operated controls.

Pharmacokinetic sample preparation and analysis. Pharmacokinetic studies were conducted by Xenobiotic Laboratories (Plainsboro, NJ). Briefly, male CD-1 mice received a single IV bolus dose of [¹⁴C] CN-105 52 µCi/mg (dose of 1.92 mg/kg, and approximately 100 µCi/kg) by tail vein injection. At 0.083, 0.5, 1, 6, 24, and 48 hours post-dose terminal blood samples were collected from 2 animals per timepoint and plasma was isolated for radiometric analysis.

To assess CNS penetration, quantitative whole body autoradiography (QWBA) analysis was performed and areas of interest in blood and brain were compared. The carcasses were frozen in a dry ice/hexane bath and embedded in low viscosity carboxymethylcellulose for sagittal, 30-µm thick sections of low viscosity carboxymethylcellulose-of mouse carcasses were sectioned. Pharmacokinetic data were generated by non-compartmental PK analysis of total radioactivity concentration vs. time profiles for tissues using WinNonlin (version 6.3, Pharsight Corporation, Mountain View, CA).

Selected tissues and areas of interest (AOI) were analyzed using QWBA. The tissues and AOI were analyzed within AIDA software using a region sampling tool. The following parameters were derived for individual subjects: maximum tissue and plasma concentration (C_{max}), time of C_{max} (t_{max}), area under the tissue concentration versus time curve from time zero to the last quantifiable tissue concentration (AUC_{0-t}), area under the tissue concentration versus time curve from time zero to 4 hours (AUC_{0-4}), and the apparent terminal half-life ($t_{1/2}$). AUC_{0-4} was calculated using the linear trapezoidal rule. Results for AOIs are expressed in nanogram equivalents of (¹⁴C) CN-105 per gram of tissue (ng equiv./g) and were calculated as follows: ng equiv./g is equal to nCi per gram of tissue per nCi/ng of (¹⁴C) CN-105.

Statistical analysis. After testing for normality of data by constructing normal probability plots with raw data, serial tests of functional performance, including Rotarod and MWM performance, were compared with a two factor repeated measures analysis of variance (ANOVA) with time as the repeated variable. When F-value was significant for group effect, pairwise comparison was performed using *post-hoc* Scheffe test for correcting multiple comparisons. The number of F4/80 and Fluoro-Jade B positive cells was compared among groups with the Kruskal–Wallis H statistic. Between groups, differences were compared by the Mann–Whitney U statistic. Parametric values are expressed as mean ± standard deviation (SD). Significance was assumed if $p < 0.05$.

References

1. Faul, M., Xu, L., Wald, M. M., Coronado, V. & Dellinger, A. M. Traumatic Brain Injury in the United States: National Estimates of Prevalence and Incidence, 2002–2006. *Injury Prev* **16**, A268–A268, doi: 10.1136/ip.2010.029951 (2010).
2. Flanagan, S. R. Invited Commentary on “Centers for Disease Control and Prevention Report to Congress: Traumatic Brain Injury in the United States: Epidemiology and Rehabilitation”. *Arch Phys Med Rehab* **96**, 1753–1755, doi: 10.1016/j.apmr.2015.07.001 (2015).
3. Selassie, A. W. *et al.* Incidence of long-term disability following traumatic brain injury hospitalization, United States, 2003. *J Head Trauma Rehab* **23**, 123–131, doi: 10.1097/01.Htr.0000314531.30401.39 (2008).
4. Zaloshnja, E., Miller, T., Langlois, J. A. & Selassie, A. W. Prevalence of Long-Term Disability From Traumatic Brain Injury in the Civilian Population of the United States, 2005. *J Head Trauma Rehab* **23**, 394–400, doi: 10.1097/01.Htr.0000341435.52004.Ac (2008).
5. Corps, K. N., Roth, T. L. & McGavern, D. B. Inflammation and neuroprotection in traumatic brain injury. *JAMA Neurol* **72**, 355–362, doi: 10.1001/jamaneurol.2014.3558 (2015).
6. Roberts, I. *et al.* Effect of intravenous corticosteroids on death within 14 days in 10008 adults with clinically significant head injury (MRC CRASH trial): randomised placebo-controlled trial. *Lancet* **364**, 1321–1328, doi: 10.1016/S0140-6736(04)17188-2 (2004).
7. McConeghy, K. W., Hatton, J., Hughes, L. & Cook, A. M. A review of neuroprotection pharmacology and therapies in patients with acute traumatic brain injury. *CNS drugs* **26**, 613–636, doi: 10.2165/11634020-000000000-00000 (2012).
8. Lanterna, L. A. *et al.* Meta-analysis of APOE genotype and subarachnoid hemorrhage: clinical outcome and delayed ischemia. *Neurology* **69**, 766–775, doi: 10.1212/01.wnl.0000267640.03300.6b (2007).
9. Morris, P. G., Wilson, J. T., Dunn, L. T. & Nicoll, J. A. Apolipoprotein E polymorphism and neuropsychological outcome following subarachnoid haemorrhage. *Acta Neurol Scand* **109**, 205–209 (2004).
10. Alberts, M. J. *et al.* ApoE genotype and survival from intracerebral haemorrhage. *Lancet* **346**, 575 (1995).
11. Biffi, A. *et al.* APOE genotype and extent of bleeding and outcome in lobar intracerebral haemorrhage: a genetic association study. *Lancet Neurol* **10**, 702–709, doi: 10.1016/S1474-4422(11)70148-X (2011).
12. Alexander, S. *et al.* Apolipoprotein E4 allele presence and functional outcome after severe traumatic brain injury. *Journal of neurotrauma* **24**, 790–797, doi: 10.1089/neu.2006.0133 (2007).
13. Kassam, I., Gagnon, F. & Cusimano, M. D. Association of the APOE-epsilon4 allele with outcome of traumatic brain injury in children and youth: a meta-analysis and meta-regression. *J Neurol Neurosurg Psychiatry* **87**, 433–440, doi: 10.1136/jnnp-2015-310500 (2016).
14. Lawrence, D. W., Comper, P., Hutchison, M. G. & Sharma, B. The role of apolipoprotein E epsilon (epsilon)-4 allele on outcome following traumatic brain injury: A systematic review. *Brain Inj* **29**, 1018–1031, doi: 10.3109/02699052.2015.1005131 (2015).
15. Li, L. *et al.* The Association Between Apolipoprotein E and Functional Outcome After Traumatic Brain Injury: A Meta-Analysis. *Medicine (Baltimore)* **94**, e2028, doi: 10.1097/MD.0000000000002028 (2015).

16. Willemse-van Son, A. H., Ribbers, G. M., Hop, W. C., van Duijn, C. M. & Stam, H. J. Association between apolipoprotein-epsilon4 and long-term outcome after traumatic brain injury. *J Neurol Neurosurg Psychiatry* **79**, 426–430, doi: 10.1136/jnnp.2007.129460 (2008).
17. Zhou, W. *et al.* Meta-analysis of APOE4 allele and outcome after traumatic brain injury. *Journal of neurotrauma* **25**, 279–290, doi: 10.1089/neu.2007.0489 (2008).
18. Laskowitz, D. T., Goel, S., Bennett, E. R. & Matthew, W. D. Apolipoprotein E suppresses glial cell secretion of TNF alpha. *Journal of neuroimmunology* **76**, 70–74 (1997).
19. Lynch, J. R. *et al.* Apolipoprotein E affects the central nervous system response to injury and the development of cerebral edema. *Annals of neurology* **51**, 113–117 (2002).
20. Linton, M. F. *et al.* Phenotypes of apolipoprotein B and apolipoprotein E after liver transplantation. *The Journal of clinical investigation* **88**, 270–281, doi: 10.1172/JCI115288 (1991).
21. Laskowitz, D. T., Fillit, H., Yeung, N., Toki, K. & Vitek, M. P. Apolipoprotein E-derived peptides reduce CNS inflammation: implications for therapy of neurological disease. *Acta neurologica Scandinavica. Supplementum* **185**, 15–20, doi: 10.1111/j.1600-0404.2006.00680.x (2006).
22. Laskowitz, D. T. *et al.* Downregulation of microglial activation by apolipoprotein E and apoE-mimetic peptides. *Experimental neurology* **167**, 74–85, doi: 10.1006/exnr.2001.7541 (2001).
23. Lynch, J. R. *et al.* APOE genotype and an ApoE-mimetic peptide modify the systemic and central nervous system inflammatory response. *The Journal of biological chemistry* **278**, 48529–48533, doi: 10.1074/jbc.M306923200 (2003).
24. James, M. L., Sullivan, P. M., Lascola, C. D., Vitek, M. P. & Laskowitz, D. T. Pharmacogenomic effects of apolipoprotein e on intracerebral hemorrhage. *Stroke; a journal of cerebral circulation* **40**, 632–639, doi: 10.1161/STROKEAHA.108.530402 (2009).
25. Gao, J. *et al.* A novel apoE-derived therapeutic reduces vasospasm and improves outcome in a murine model of subarachnoid hemorrhage. *Neurocritical care* **4**, 25–31, doi: 10.1385/NCC.4:1:025 (2006).
26. Mesis, R. G. *et al.* Dissociation between vasospasm and functional improvement in a murine model of subarachnoid hemorrhage. *Neurosurgical focus* **21**, E4 (2006).
27. Hoane, M. R. *et al.* The novel apolipoprotein E-based peptide COG1410 improves sensorimotor performance and reduces injury magnitude following cortical contusion injury. *Journal of neurotrauma* **24**, 1108–1118, doi: 10.1089/neu.2006.0254 (2007).
28. Hoane, M. R., Kaufman, N., Vitek, M. P. & McKenna, S. E. COG1410 improves cognitive performance and reduces cortical neuronal loss in the traumatically injured brain. *Journal of neurotrauma* **26**, 121–129, doi: 10.1089/neu.2008.0565 (2009).
29. Laskowitz, D. T. *et al.* Traumatic brain injury exacerbates neurodegenerative pathology: improvement with an apolipoprotein E-based therapeutic. *Journal of neurotrauma* **27**, 1983–1995, doi: 10.1089/neu.2010.1396 (2010).
30. Lynch, J. R. *et al.* A novel therapeutic derived from apolipoprotein E reduces brain inflammation and improves outcome after closed head injury. *Experimental neurology* **192**, 109–116, doi: 10.1016/j.expneurol.2004.11.014 (2005).
31. Hoe, H. S., Harris, D. C. & Rebeck, G. W. Multiple pathways of apolipoprotein E signaling in primary neurons. *Journal of neurochemistry* **93**, 145–155, doi: 10.1111/j.1471-4159.2004.03007.x (2005).
32. Misra, U. K. *et al.* Apolipoprotein E and mimetic peptide initiate a calcium-dependent signaling response in macrophages. *Journal of leukocyte biology* **70**, 677–683 (2001).
33. Pocivavsek, A., Burns, M. P. & Rebeck, G. W. Low-density lipoprotein receptors regulate microglial inflammation through c-Jun N-terminal kinase. *Glia* **57**, 444–453, doi: 10.1002/glia.20772 (2009).
34. Pocivavsek, A., Mikhailenko, I., Strickland, D. K. & Rebeck, G. W. Microglial low-density lipoprotein receptor-related protein 1 modulates c-Jun N-terminal kinase activation. *Journal of neuroimmunology* **214**, 25–32, doi: 10.1016/j.jneuroim.2009.06.010 (2009).
35. Cernak, I. *et al.* The pathobiology of moderate diffuse traumatic brain injury as identified using a new experimental model of injury in rats. *Neurobiology of disease* **17**, 29–43, doi: 10.1016/j.nbd.2004.05.011 (2004).
36. Morris, R. Developments of a water-maze procedure for studying spatial learning in the rat. *Journal of neuroscience methods* **11**, 47–60 (1984).
37. Yu, T. S., Zhang, G., Liebl, D. J. & Kernie, S. G. Traumatic brain injury-induced hippocampal neurogenesis requires activation of early nestin-expressing progenitors. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **28**, 12901–12912, doi: 10.1523/JNEUROSCI.4629-08.2008 (2008).
38. Iyer, S. S. & Cheng, G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Crit Rev Immunol* **32**, 23–63 (2012).
39. Yuan, X., Peng, X., Li, Y. & Li, M. Role of IL-38 and its related cytokines in inflammation. *Mediators Inflamm* **2015**, 807976, doi: 10.1155/2015/807976 (2015).
40. van de Veerdonk, F. L. *et al.* IL-38 binds to the IL-36 receptor and has biological effects on immune cells similar to IL-36 receptor antagonist. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 3001–3005, doi: 10.1073/pnas.1121534109 (2012).
41. Mora, J. *et al.* Interleukin-38 is released from apoptotic cells to limit inflammatory macrophage responses. *J Mol Cell Biol*, doi: 10.1093/jmcb/mjw006 (2016).
42. Can, A. *et al.* Antidepressant-like responses to lithium in genetically diverse mouse strains. *Genes Brain Behav* **10**, 434–443, doi: 10.1111/j.1601-183X.2011.00682.x (2011).
43. Macleod, J. N., Sorensen, M. P. & Shapiro, B. H. Strain independent elevation of hepatic mono-oxygenase enzymes in female mice. *Xenobiotica* **17**, 1095–1102 (1987).
44. Han, S. H. & Chung, S. Y. Marked hippocampal neuronal damage without motor deficits after mild concussive-like brain injury in apolipoprotein E-deficient mice. *Annals of the New York Academy of Sciences* **903**, 357–365 (2000).
45. Aono, M. *et al.* Protective effect of apolipoprotein E-mimetic peptides on N-methyl-D-aspartate excitotoxicity in primary rat neuronal-glial cell cultures. *Neuroscience* **116**, 437–445 (2003).
46. Laskowitz, D. T. *et al.* COG1410, a novel apolipoprotein E-based peptide, improves functional recovery in a murine model of traumatic brain injury. *Journal of neurotrauma* **24**, 1093–1107, doi: 10.1089/neu.2006.0192 (2007).
47. Qiu, Z., Crutcher, K. A., Hyman, B. T. & Rebeck, G. W. ApoE isoforms affect neuronal N-methyl-D-aspartate calcium responses and toxicity via receptor-mediated processes. *Neuroscience* **122**, 291–303 (2003).
48. Sheng, Z., Prorok, M., Brown, B. E. & Castellino, F. J. N-methyl-D-aspartate receptor inhibition by an apolipoprotein E-derived peptide relies on low-density lipoprotein receptor-associated protein. *Neuropharmacology* **55**, 204–214, doi: 10.1016/j.neuropharm.2008.05.016 (2008).
49. Fan, L. *et al.* Experimental brain injury induces differential expression of tumor necrosis factor-alpha mRNA in the CNS. *Brain Res Mol Brain Res* **36**, 287–291 (1996).
50. Kinoshita, K. *et al.* Interleukin-1beta messenger ribonucleic acid and protein levels after fluid-percussion brain injury in rats: importance of injury severity and brain temperature. *Neurosurgery* **51**, 195–203, discussion 203 (2002).
51. Szymdynger-Chodobska, J., Strazielle, N., Zink, B. J., Ghersi-Egea, J. F. & Chodobski, A. The role of the choroid plexus in neutrophil invasion after traumatic brain injury. *J Cereb Blood Flow Metab* **29**, 1503–1516, doi: 10.1038/jcbfm.2009.71 (2009).
52. Bergold, P. J. Treatment of traumatic brain injury with anti-inflammatory drugs. *Experimental neurology* **275** Pt 3, 367–380, doi: 10.1016/j.expneurol.2015.05.024 (2016).
53. Woodcock, T. & Morganti-Kossmann, M. C. The role of markers of inflammation in traumatic brain injury. *Front Neurol* **4**, 18, doi: 10.3389/fneur.2013.00018 (2013).

54. Longhi, L. *et al.* Effect of traumatic brain injury on cognitive function in mice lacking p55 and p75 tumor necrosis factor receptors. *Acta Neurochir Suppl* **102**, 409–413 (2008).
55. Scherbel, U. *et al.* Differential acute and chronic responses of tumor necrosis factor-deficient mice to experimental brain injury. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 8721–8726 (1999).
56. Stahel, P. F. *et al.* Experimental closed head injury: analysis of neurological outcome, blood-brain barrier dysfunction, intracranial neutrophil infiltration, and neuronal cell death in mice deficient in genes for pro-inflammatory cytokines. *J Cereb Blood Flow Metab* **20**, 369–380, doi: 10.1097/00004647-200002000-00019 (2000).
57. Shohami, E., Ginis, I. & Hallenbeck, J. M. Dual role of tumor necrosis factor alpha in brain injury. *Cytokine & growth factor reviews* **10**, 119–130 (1999).
58. Ziebell, J. M. & Morganti-Kossmann, M. C. Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury. *Neurotherapeutics* **7**, 22–30, doi: 10.1016/j.nurt.2009.10.016 (2010).
59. Li, G. Z. *et al.* Expression of myeloid differentiation primary response protein 88 (Myd88) in the cerebral cortex after experimental traumatic brain injury in rats. *Brain Res* **1396**, 96–104, doi: 10.1016/j.brainres.2011.04.014 (2011).
60. Li, W. *et al.* Enhanced cortical expression of myeloid differentiation primary response protein 88 (Myd88) in patients with traumatic brain injury. *J Surg Res* **180**, 133–139, doi: 10.1016/j.jss.2012.10.928 (2013).
61. Koedel, U. *et al.* Acute brain injury triggers MyD88-dependent, TLR2/4-independent inflammatory responses. *The American journal of pathology* **171**, 200–213, doi: 10.2353/ajpath.2007.060821 (2007).
62. Gonias, S. L. & Campana, W. M. LDL receptor-related protein-1: a regulator of inflammation in atherosclerosis, cancer, and injury to the nervous system. *The American journal of pathology* **184**, 18–27, doi: 10.1016/j.ajpath.2013.08.029 (2014).
63. Bell, R. D. *et al.* Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. *Nature* **485**, 512–516, doi: 10.1038/nature11087 (2012).
64. Croy, J. E., Brandon, T. & Komives, E. A. Two apolipoprotein E mimetic peptides, ApoE(130–149) and ApoE(141–155)2, bind to LRP1. *Biochemistry* **43**, 7328–7335, doi: 10.1021/bi036208p (2004).
65. Ophir, G. *et al.* Human apoE3 but not apoE4 rescues impaired astrocyte activation in apoE null mice. *Neurobiology of disease* **12**, 56–64 (2003).
66. Chen, G. Y. & Nunez, G. Sterile inflammation: sensing and reacting to damage. *Nature reviews. Immunology* **10**, 826–837, doi: 10.1038/nri2873 (2010).
67. Yu, L., Wang, L. & Chen, S. Endogenous toll-like receptor ligands and their biological significance. *Journal of cellular and molecular medicine* **14**, 2592–2603, doi: 10.1111/j.1582-4934.2010.01127.x (2010).
68. Said-Sadier, N. & Ojcius, D. M. Alarmins, inflammasomes and immunity. *Biomedical journal* **35**, 437–449, doi: 10.4103/2319-4170.104408 (2012).
69. Westermarck, G. T., Westermarck, P., Berne, C., Korsgren, O. & Nordic Network for Clinical Islet, T. Widespread amyloid deposition in transplanted human pancreatic islets. *The New England journal of medicine* **359**, 977–979, doi: 10.1056/NEJMc0802893 (2008).
70. Stewart, C. R. *et al.* CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nature immunology* **11**, 155–161, doi: 10.1038/ni.1836 (2010).
71. Barish, G. D. *et al.* Bcl-6 and NF-kappaB cistromes mediate opposing regulation of the innate immune response. *Genes & development* **24**, 2760–2765, doi: 10.1101/gad.1998010 (2010).
72. Barish, G. D. *et al.* The Bcl6-SMRT/NCOR cistrome represses inflammation to attenuate atherosclerosis. *Cell metabolism* **15**, 554–562, doi: 10.1016/j.cmet.2012.02.012 (2012).
73. Basso, K. *et al.* Integrated biochemical and computational approach identifies BCL6 direct target genes controlling multiple pathways in normal germinal center B cells. *Blood* **115**, 975–984, doi: 10.1182/blood-2009-06-227017 (2010).
74. Lei, B. *et al.* Neuroprotective pentapeptide CN-105 improves functional and histological outcomes in a murine model of intracerebral hemorrhage. *Sci Rep* **6**, 34834, doi: 10.1038/srep34834 (2016).
75. Nishitsuji, K., Hosono, T., Nakamura, T., Bu, G. & Michikawa, M. Apolipoprotein E regulates the integrity of tight junctions in an isoform-dependent manner in an *in vitro* blood-brain barrier model. *The Journal of biological chemistry* **286**, 17536–17542, doi: 10.1074/jbc.M111.225532 (2011).
76. Weisgraber, K. H. Apolipoprotein E: structure-function relationships. *Advances in protein chemistry* **45**, 249–302 (1994).
77. Marklund, N. & Hillered, L. Animal modelling of traumatic brain injury in preclinical drug development: where do we go from here? *Br J Pharmacol* **164**, 1207–1229, doi: 10.1111/j.1476-5381.2010.01163.x (2011).
78. Petraglia, A. L., Dashnaw, M. L., Turner, R. C. & Bailes, J. E. Models of Mild Traumatic Brain Injury: Translation of Physiological and Anatomic Injury. *Neurosurgery* **75**, S34–S49, doi: 10.1227/NEU.0000000000000472 (2014).
79. Wang, H. *et al.* ApolipoproteinE mimetic peptides improve outcome after focal ischemia. *Experimental neurology* **241**, 67–74, doi: 10.1016/j.expneurol.2012.11.027 (2013).
80. Amor, S. & Woodroffe, M. N. Innate and adaptive immune responses in neurodegeneration and repair. *Immunology* **141**, 287–291, doi: 10.1111/imm.12134 (2014).
81. Le Thuc, O., Blondeau, N., Nahon, J. L. & Rovere, C. The complex contribution of chemokines to neuroinflammation: switching from beneficial to detrimental effects. *Annals of the New York Academy of Sciences* **1351**, 127–140, doi: 10.1111/nyas.12855 (2015).
82. Dawson, H. N., Cantillana, V., Chen, L. & Vitek, M. P. The tau N279K exon 10 splicing mutation recapitulates frontotemporal dementia and parkinsonism linked to chromosome 17 tauopathy in a mouse model. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **27**, 9155–9168, doi: 10.1523/JNEUROSCI.5492-06.2007 (2007).
83. Hamm, R. J., Pike, B. R., O'Dell, D. M., Lyeth, B. G. & Jenkins, L. W. The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. *Journal of neurotrauma* **11**, 187–196 (1994).

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Author Contributions

D.T. La, H.W. and H.N.D. were involved in conceiving of the project, experimental design, analyzing the data, preparing figures, and writing the manuscript. H.W. oversaw and executed the TBI animal modeling. T.M.T. executed sham experiments and peptide injections. D.T. Lu and V.C. performed behavioral studies, tissue collection and immunohistochemistry. T.C., G.Z., and G.M. prepared animal tissue for RNA extraction and performed immunohistochemistry. D.K. conducted the gene expression experiments. H.N.D. performed cell quantification and image analysis. D.T. La, H.N.D., and B.J.K. performed the statistical analysis. B.J.K. contributed to experimental design and critical review of the manuscript.

Additional Information

Competing Interests: The patent for CN-105 is held by Duke University, and D.T. La, B.J.K., and H.N.D. are co-inventors. D.T. La is an officer of Aegis-CN, which supplied drug for these studies, but had no role in experimental design or writing of this manuscript.

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Phase I Randomized, Double-Blind, Placebo-Controlled Study to Determine the Safety, Tolerability, and Pharmacokinetics of a Single Escalating Dose and Repeated Doses of CN-105 in Healthy Adult Subjects

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Abstract

Spontaneous intracranial hemorrhage (ICH) remains a devastating stroke subtype, affecting as many as 80,000 people annually in the United States and associated with extremely high mortality. In the absence of any pharmacological interventions demonstrated to improve outcome, care for patients with ICH remains largely supportive. Thus, despite advances in the understanding of ICH and brain injury, there remains an unmet need for interventions that improve neurologic recovery and outcomes. Recent research suggesting inflammation and APOE genotype play a role in modifying neurologic outcome after brain injury has led to the development of an APOE-derived peptide agent (CN-105). Preclinical studies have demonstrated that CN-105 effectively downregulates the inflammatory response in acute brain injury, including ICH. Following Investigational New Drug (IND) enabling studies in murine models, this first-in-human single escalating dose and multiple dose placebo-controlled clinical trial was performed to define the safety and pharmacokinetics (PK) of CN-105. A total of 48 subjects (12 control, 36 active) were randomized in this study; all subjects completed the study. No significant safety issues were identified with both dosing regimens, and PK analysis revealed linearity without significant drug accumulation. The median half-life in the terminal elimination phase of CN-105 following a single or repeated dosing regimen did not change (approximately 3.6 hours). With the PK and preliminary safety of CN-105 established, the drug is now poised to begin first-in-disease phase 2 clinical trials in patients with ICH who urgently need new therapeutic options.

Keywords

intracerebral hemorrhage, brain injury, neuroinflammation, neuroprotection, apolipoprotein E, pharmacokinetics

Acute brain injury resulting from cerebrovascular diseases and trauma is associated with extremely high mortality and morbidity.^{1,2} Despite advances in our understanding of basic cellular and molecular mechanisms associated with primary and secondary neuronal injury, no effective neuroprotective pharmacological interventions have improved functional outcomes.^{3,4} Supportive medical care remains the mainstay of management for patients with acute brain injury, and mortality rates for many of these injuries have not improved in the last 2 decades.^{2,5,6} There is a clear and urgent unmet clinical need for neuroprotective therapeutics for acute brain injury.

Traditionally, new therapeutic strategies have focused on our increased mechanistic understanding of disease-specific pathophysiology. However, despite these important disease-specific differences, the neuroinflammatory response, characterized by glial activation and release of mediators of inflammation, neuronal excitotoxicity, and oxidative stress, serves as a common denominator that exacerbates secondary

neuronal injury in a variety of acute and chronic neuropathology.⁷ Moreover, in the setting of acute brain injury, neuroinflammation plays an important role in mediating tissue injury for days after the initial insult^{8–10}; this has the potential to lengthen the therapeutic window as compared to strategies that solely target excitotoxicity. Thus, a therapeutic strategy that targets maladaptive neuroinflammatory responses holds promise in the treatment of diverse forms of brain injury.

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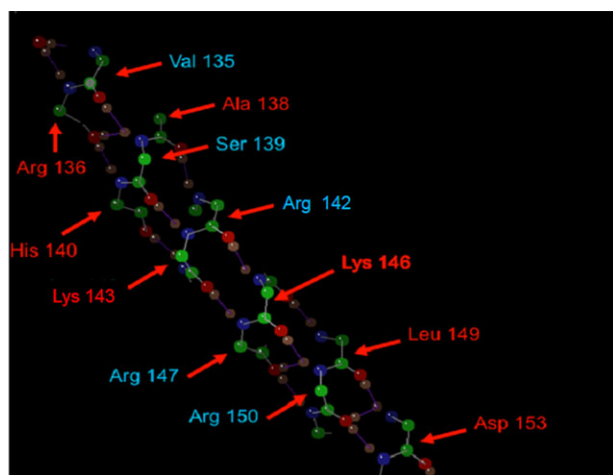


Figure 1. Amino acid structure of the receptor-binding region of ApoE. CN-105 is a pentapeptide consisting of the residues identified in blue.

Apolipoprotein E (apoE) is an endogenous brain protein synthesized in response to brain injury and exists in humans as 3 common protein isoforms, designated apoE2, apoE3, and apoE4, which differ by single amino acid substitutions at residues 112 and 158.¹¹ Isoform-specific protective effects on endogenous neuroinflammatory responses have been observed in humans^{12,13} and demonstrated in preclinical models of brain injury.^{14–16} Mechanisms by which apoE reduces neurologic inflammation have been demonstrated through specific receptor interactions^{17–19} and down-regulation of microglial activation.^{20,21}

Previous studies from our lab and others have demonstrated that apoE, a 299aminoacid protein produced within the brain, modifies neuroinflammatory responses by downregulating glial activation and release of inflammatory mediators. However, apoE holoprotein's therapeutic potential is limited, as it does not cross the blood-brain barrier (BBB). To address this limitation, we have developed apoE-mimetic peptides derived from the helical receptor binding region of apoE. These peptides have been demonstrated to improve histological and long-term functional outcome in preclinical models of intracerebral hemorrhage (ICH),^{22,23} traumatic brain injury,^{24–27} ischemic stroke,^{28,29} subarachnoid hemorrhage,^{30,31} and spinal cord injury.^{32,33}

Recently, we have selected CN-105, an apoE-mimetic pentapeptide derived from the receptor-binding face of apoE (Figure 1), as the lead candidate for further development based on its profile in *in vitro* cell culture models of neuroinflammation and neuroprotection, *in vivo* efficacy in preclinical models of intracranial hemorrhage,³⁴ stroke, and acute brain injury and preclinical safety profile.³⁴ Results from rodent and beagle Good Laboratory Practice safety and toxicology

studies demonstrated that repeated intravenous administration of CN-105 at 5 mg/kg doses (100-fold the therapeutic dose identified in preclinical efficacy studies), 4 times per day (20 mg/kg per day) for 14 days was well tolerated, and 20 mg/kg per day was identified as the no-observed-adverse effect level (NOAEL) in these studies. Based on these promising preclinical results, the decision was made to further develop CN-105 for the treatment of human acute brain injuries.

CN-105 has never been evaluated in humans, its safety and pharmacokinetics (PK) have not been defined, and the current first-in-human (FIH) study represents the first clinical translation of this molecule to a clinical population.

Methods

The protocol was approved by the FDA under an Investigational New Drug Application and by the Duke University Institutional Review Board. Written informed consent was obtained for each volunteer prior to performing study-related procedures.

Eligibility Criteria

Healthy male and female volunteers aged 18 to 50 with BMI ranging from 18 to 33 kg/m² and weight of at least 50 kg were eligible for the study. Volunteers were required to have adequate peripheral vein access, no exposure to prescription medication (except contraception) or over the counter (OTC) medications or herbal/vitamin supplements (except acetaminophen ≤ 1 g/day and stable, nonglucocorticoid treatment of seasonal allergies) in the 7 days prior to study entry, no exposure to nicotine-containing products for ≥ 6 months, no current or recent (within 2 years) history of alcohol or drug abuse, caffeine consumption ≤ 3 cups of coffee per day, ability to comply with medically acceptable contraception or prior history of surgical sterilization, and no history of recent (30 days cellular and 90 days acellular) blood donation. Pregnant or lactating females and volunteers with significant medical or psychiatric illness by history or examination that would influence study results or preclude informed consent and study compliance were excluded.

Study Design

This was a phase 1, single-center, randomized, double-blind, placebo-controlled study to determine the safety, tolerability, and pharmacokinetics (PK) of a single ascending dose (SAD) and repeated doses of intravenous CN-105 in healthy adults. In the SAD portion of this FIH study, 8 participants were randomized to CN-105 or saline control (6 active, 2 control) at 0.01, 0.03, 0.1, 0.3, and 1.0 mg/kg administered over 30 minutes. Intermittent weight-based dosing was supported by preclinical animal models and was considered

more practical in a clinical setting than a continuous intravenous dose.³⁴ The maximum dose in the study was the highest dose allowed by the US Food and Drug Administration (FDA). Because this was a FIH study, an interim PK analysis was planned during the SAD portion of the study to determine the optimal PK sampling time points. In the repeat-dose cohort 8 participants (6 active, 2 control) were randomized to receive repeated infusions of CN-105 over 30 minutes every 6 hours for 72 hours.

Data Monitoring Committee

A data-monitoring committee was used to evaluate safety and tolerability with the dose escalation cohorts and to select the final dose for the repeated-dose cohort. Doses were escalated in successive cohorts unless 1 participant experienced an adverse effect of severe grade or 2 participants reported the same adverse effect of moderate intensity that was considered probably related to the study drug.

Safety Evaluations

During and following dosing, safety and tolerability endpoints included reported adverse events (AEs), changes in vital signs, physical examination findings, ECG, and clinical laboratory tests (hematologic, chemistry, urinalysis).

Pharmacokinetic Sampling

During the SAD cohorts, blood samples were taken at 15 minutes prior to start of dosing and at 0.083, 0.167, 0.5, 1, 2, 4, 8, 12, and 24 hours from the start of dosing. Urine samples were obtained prior to dose administration, and then pooled samples (0-3, 3-6, 6-8, 8-12, 12-24 hours) were collected from the start of dosing. Interim PK analysis after the second (0.03 mg/kg) cohort resulted in removal of a 168-hour blood sample and addition of a urine sample from 8-12 hours. For repeat dose participants, blood samples were taken 15 minutes prior to start of dosing and then at 0.083, 0.167, 0.5, 1, 2, 4, 5 to 6 hours for the first 2 doses and within 1 hour prior to each dose thereafter. Blood samples were also collected at 0.083, 0.167, 0.5, 1, 2, 4, 5 to 6, and 12 hours after the last dose.

Plasma and urine drug concentrations were determined by MPI (Mattawan, Michigan). Plasma samples were stored in 1% HALT with K₂EDTA at -70°C before they were ready to be analyzed. Liquid chromatography-tandem mass spectrometry analysis was performed using a positive Turbo IonSpray® interface on a Sciex API-3000 (Applied Biosystems, Foster City, California) and multiple reaction monitoring. The analytical range was 1.00 ng/mL to 1000 ng/mL. The assay precision in quality-control samples was <20%

for the lowest limit of quantitation and < 15% for all other concentrations.

Pharmacokinetics and Safety Analysis

Individual plasma concentration vs time profiles of CN-105 for SAD and repeat doses were used to generate PK parameters using noncompartmental analysis. The noncompartmental PK analysis was performed in Phoenix WinNonLin (version 6.3, Pharsight Corporation, St. Louis, Missouri) using concentration vs time data obtained for CN-105. All plasma concentrations below the quantitation limit were assigned a value of 0. For both SAD and repeat-dose cohorts, the peak drug concentration (C_{max}) and time of peak concentration (T_{max}) were obtained from the observed data. For the SAD cohort, AUC from 0 to last measurable concentration (AUC_{0-last}) and AUC from 0 to infinity ($AUC_{0-\infty}$) and elimination half-life ($t_{1/2}$) were calculated. For the repeat-dose cohort, AUC from 0 to 6 hours (AUC_{τ}) was assessed for CN-105. AUC_{0-last} and AUC_{τ} were calculated using the trapezoidal rule. In addition, the $AUC_{0-\infty}$ was calculated from $AUC_{0-last} + Ct/\lambda_z$ where Ct is the last measurable concentration, and λ_z is the terminal elimination rate constant calculated by fitting 3 points to a linear regression. The half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$. At least 3 time points with measurable plasma concentrations were required for the calculation of AUC_{last} , and at least 3 time points (of which the first time point must be greater than T_{max}) with measurable plasma concentrations were required for the calculation of λ_z . Total body clearance (CL) was calculated as $dose/AUC_{\infty}$. The volume of distribution (V) was calculated as $V = CL/\lambda_z$.

Primary analysis of safety and tolerability using vital signs, ECG, clinical laboratory results, and AEs was performed using descriptive statistics. Categorical variables were analyzed as counts and percentages, while continuous variables were presented as means and standard deviations or medians and interquartile ranges. Statistical analyses were performed using Version 9.4 (or newer) of SAS® (Cary, North Carolina) on a Unix operating system or Stata 13.1 (College Station, Texas).

Results

Demographics

Sixty-six adults were enrolled, and 48 completed the clinical trial. The study population was predominantly male (79%) and black (69%) (Table 1). These demographics were similar between the placebo and treatment groups.

Safety and Tolerability

Among the 48 subjects in this study, 23 (47%; 18 active; 5 placebo) experienced an AE. A total of 18

Table 1. Characteristics of Enrolled Subjects

	Placebo (N = 12)	CN-105 (N = 36)	Total (N = 48)
Age, median (Q1, Q3)	32.0 (28.9, 41.0)	32.4 (28.3, 37.2)	32.4 (28.3, 37.6)
Sex			
Male	9	29	38
Female	3	7	10
Race			
Black	7	26	33
White	3	7	10
Other	2	3	5
BMI, median (Q1, Q3)	27.6 (25.1, 30.7)	26.3 (24.0, 28.4)	26.6 (24.0, 29.2)

N, number; Q, quartile; BMI, body mass index.

Table 2. Pharmacokinetic Values for Single-Dose CN-105

Parameter ^a	Dose				
	0.01 mg/kg	0.03 mg/kg	0.1 mg/kg	0.3 mg/kg	1.0 mg/kg
C _{max} (ng/mL)	35.7 ± 10.9	107.5 ± 25.5	407.8 ± 128.9	965.8 ± 209.3	3943.3 ± 507.0
AUC _{0-∞} (ng · h/mL)	95.9 ± 20.2	280.8 ± 39.6	1126.1 ± 194.5	2672.3 ± 272.3	9548 ± 1281.2
Volume (L)	29.8 ± 3.2	30.4 ± 8.0	38.9 ± 12.0	45.6 ± 11.2	38.4 ± 8.1
Clearance (L/h)	9.0 ± 1.1	9.0 ± 1.1	8.2 ± 1.9	8.8 ± 1.5	7.7 ± 1.7
T _{1/2}	2.3 ± 0.5	2.4 ± 0.5	3.3 ± 0.6	3.6 ± .4	3.5 ± 0.3

AUC_{0-∞}, area under the plasma concentration-time curve from 0 to infinity; CL, clearance; C_{max}, maximum plasma drug concentration; t_{1/2}, half-life.

^aData provided as mean ± SD.

subjects (37.5%) experienced a treatment-emergent AE, 4 (33.3%) in the placebo group and 14 (38.9%) in the CN-105 group. Bradycardia and headache were the most common treatment-emergent AEs. A total of 6 (12.5%) subjects experienced bradycardia, 2 (17%) in the placebo group and 4 (11%) in the CN-105 group; a total of 2 (4%) subjects reported headache, 0 (0%) in the placebo group and 2 (6%) in the CN-105 group. The bradycardia in most cases was not treatment emergent and likely related to the study population of young healthy volunteers. No serious AEs or deaths occurred. No concerning changes were observed in serial ECG, vital signs, or clinical laboratory tests.

Pharmacokinetic Summary

All subjects receiving CN-105 (n = 36) had evaluable PK data, and there were no missing data. For the single ascending doses, exposure and PK parameters are summarized in Table 2. Following IV infusions, concentrations of CN-105 in plasma declined in a polyphasic manner, as shown in the mean concentration of drug vs time plot (Figure 2). A short distribution phase was seen immediately postinfusion. A dominant β phase characterized much of the profile and exhibited log-linear behavior. At higher doses, an additional longer γ phase was present postdose at low concentrations. PK parameters for the single ascending dose regimens are summarized in Table 2. The volume, clearance, and half-life remained relatively constant over the range of doses evaluated. The mean of C_{max} and

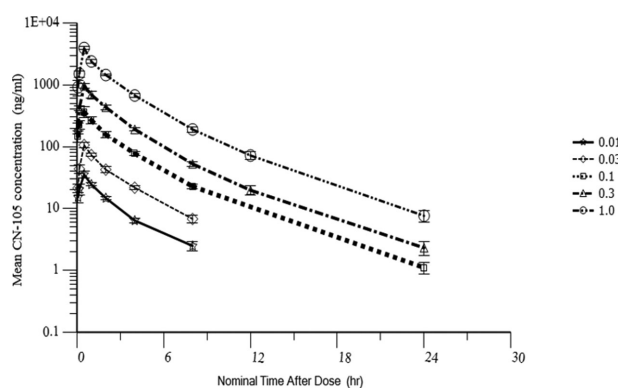


Figure 2. Concentration vs time plot for single doses of CN-105. The Y axis represents mean concentrations (ng/mL) following a single dose of CN-105, and the X axis shows the nominal time (hours) after the dose. The concentration exhibits log-linear behavior.

AUC parameters plotted vs dose in Figure 3A and 3B were well represented by linear regression lines ($r^2 > 0.99$) and consistent with dose proportionality.

PK parameters for the multiple-dosing regimen are summarized in Table 3. The median (range) terminal elimination half-life after the 13th dose was 3.6 hours (3.4-7.1). There was no significant accumulation of CN-105 as evidenced by the relatively constant AUC_τ ratios between the time points (Table 4), which is shown graphically in Figure 4. Trough concentrations reached a stable plateau by 20 hours (Figure 4), and a steady state was achieved within the first 24 hours.

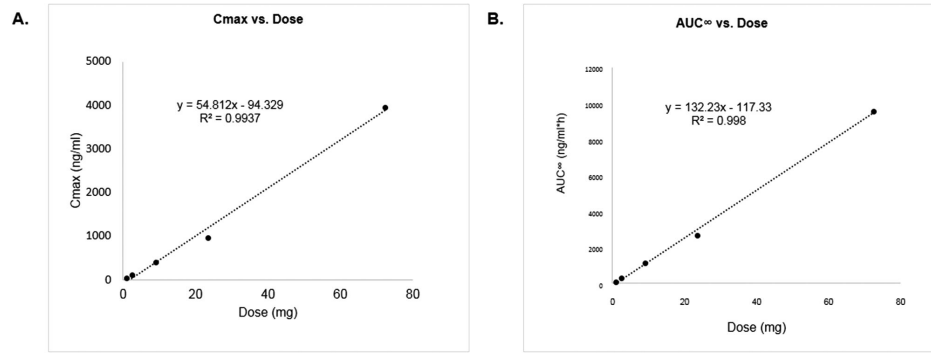


Figure 3. Linear regression demonstrates dose proportionality. C_{\max} (ng/mL) (A) and AUC_{∞} (ng · h/mL) (B) are shown on the Y axis with ascending doses of CN-105 (mg) on the X axis.

Table 3. Pharmacokinetic Values for Repeated-Dose CN-105 (1 mg/kg)

Dose Number	C_{\max} (ng/mL)	AUC_{τ} (0-6) (ng · h/mL)
1 (0 hour)	5011.67 (814.01)	9669.2 (1724.1)
2 (6 hour)	5340 (568.44)	10 557.9 (1158.5)
13 (72 hours)	5505 (404.17)	11 434.6 (1721.2)

Discussion

This phase 1, single-center, randomized, double-blind, placebo-controlled study is the FIH trial of the apoE-mimetic peptide CN-105. In this trial we have demonstrated the safety and favorable PK profile of IV CN-105 in healthy adult volunteers. We found that the CL and half-life of CN-105 were relatively consistent across doses within and between subjects and that the PK data of CN-105 administered as single IV doses between 0.01 and 1.0 mg/kg are consistent with linearity. Furthermore, a dose proportionality analysis of the linear regression of the log area under the plasma drug concentration-time curve vs the log total dose suggests that the doses were proportional to various concentrations; hence, CN-105 exposure increases proportionally after a single dose. The median half-life in the terminal elimination phase of CN-105 following a single high dose (0.1, 0.3, and 1.0 mg/kg) and after the last (13th) of a repeated dosing regimen showed that half-life (3.5 hours and 3.6 hours, respectively) did not change with multiple doses.

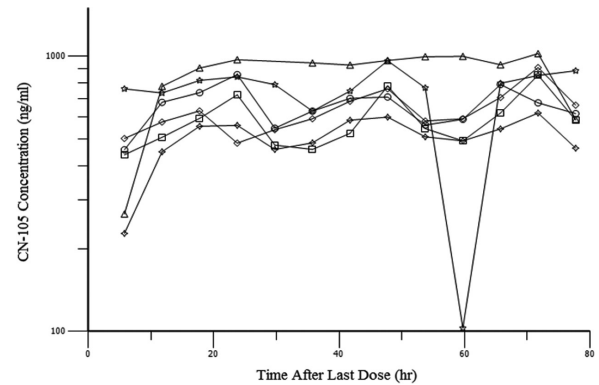


Figure 4. Concentration vs time plot following multiple doses of CN-105. Individual trough concentrations of CN-105 (ng/mL) are displayed on the Y axis with the corresponding time point on the X axis (hours). Repeated dosing of CN-105 every 6 hours achieved steady state within 24 hours and was maintained without significant drug accumulation. The extreme low steady-state value for 1 subject is likely a spurious value.

The observed half-life of 3.6 hours at higher doses in this study was longer than predicted by animal modeling (30 minutes).³⁴ This highlights the difference between human and murine pharmacokinetics and the importance of the study in determining the optimal dosing interval for human subjects. The longer half-life is of practical significance because it permits a repeated dosing regimen rather than a continuous infusion, thereby facilitating emergent administration in the clinical setting. Prior murine studies demonstrated that

Table 4. PK Parameters for Repeated Doses of CN-105 Administered as 1 mg/kg IV Infusions Over 30 Minutes

Subject ID	Dose	AUC_{τ} 1	AUC_{τ} 2	Ratio 2/1	AUC_{τ} 13	Ratio 13/1
Mean	85	9669.2	10 557.9	1.1	11 434.6	1.2
Geometric mean	84.3	9536.1	10 506.5	1.1	11 328.6	1.2
SD	11.9	1724.1	1158.5	0.2	1721.2	0.2
CV%	14	17.8	11		15.1	
Median	86	9911.7	10 448.8	1.1	11 600.4	1.3
Min	69.5	7425.1	8990.5	0.9	9382.1	0.9
Max	98.8	11 431.3	12 581.6	1.4	14 203.2	1.5

a single weight-based bolus dose achieved adequate CNS concentrations to produce long-term functional improvement, suggesting a pharmacokinetic-pharmacodynamic dissociation that makes continuous exposure unnecessary.³⁴ Additionally, the half-life of CN-105 supports the preplanned dosing paradigm of repeated intravenous administration every 6 hours for 72 hours. This dosing interval was extrapolated from cellular events that lead to peak cerebral edema and secondary neuronal injury. Of note, CN-105 shows minimal accumulation after repeated 6-hour IV doses, and steady state is achieved in the first 24 hours. Thus, CN-105 has favorable clinical pharmacological properties including a predictable linear PK and dose proportionality and a sufficiently long half-life to permit intermittent dosing. Such intermittent dosing is also more practical within a critical care setting where a patient may be receiving multiple drugs that cannot be infused simultaneously due to diluent or drug-drug interactions.

Although apoE-mimetic peptides have demonstrated robust benefits in preclinical models of acute (traumatic) brain injury,^{22–33} spontaneous (nontraumatic) ICH may be the optimal initial therapeutic target for a number of strategic reasons. Epidemiologically, ICH is a deadly condition that lacks effective treatment options and carries a high rate of morbidity and mortality¹ that has not improved in the last 20 years.⁵ To date, multiple surgical clinical trials in ICH have failed to show any significant benefit,^{35,36} and despite further ongoing surgical trials, a paradigm shift toward alternative therapies targeting other mechanisms of neuronal injury may be necessary. ICH is a clinically devastating condition with an urgent need for an alternative therapeutic entity; this makes it an attractive initial clinical outlet for pharmacological interventions designed to reduce neuroinflammatory responses. It is logistically feasible because patients present acutely and are cared for in the controlled environment of a neurointensive care or step-down unit.⁶ Unlike TBI, patients are more likely to be accompanied by a legally authorized representative, facilitating recruitment. As opposed to ischemic stroke, in which a core area of brain tissue may undergo early irreversible injury, secondary neuronal injury, such as progressive cerebral edema and mass effect, following ICH tends to occur over a more protracted period, raising the possibility of a prolonged therapeutic window.¹⁰ Furthermore, clinically relevant radiographic surrogates (brain computed tomography) are readily available and standardized to accurately localize and quantify hematoma size and to characterize the evolution of perihematomal cytotoxic edema and the resultant mass effect.³⁷ These radiographic measures of mass effect and serum biochemical markers of glial activation, neuronal injury,

and neuroinflammation serve as important objective measures to capture target engagement in the initial trials of CN-105 in patients with ICH. Phase 2 trial design for ICH is also facilitated by the availability of clinically validated prognostic scoring systems based on hemorrhage size,³⁸ location, neurological exam, and patient characteristics.³⁹ These considerations are particularly important given the absence of any previous successful studies in this area.

Conclusion

CN-105 is the leading candidate in the class of apoE-mimetic peptides derived from the receptor-binding region with the potential to improve outcomes in ICH patients when compared to the current standard of care. This phase 1 FIH study demonstrated a linear and predictable pharmacokinetic profile. Likewise, the safety profile was reassuring, demonstrating only mild, transient adverse effects. Taken together, this favorable PK and safety profile, the wealth of preclinical data demonstrating a histologically and functionally therapeutic benefit across different models of acute brain injury, and the current clinical need for better therapeutic options for ICH patients support further development of CN-105 for treatment of this population.

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Disclosures

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References

1. Mozaffarian D, Benjamin EJ, Go AS, et al. Heart disease and stroke statistics-2015 update: a report from the American Heart Association. *Circulation*. 2015;131(4):e29–e322.
2. Roozenbeek B, Maas AI, Menon DK. Changing patterns in the epidemiology of traumatic brain injury. *Nat Rev Neurol*. 2013;9(4):231–236.

3. Stocchetti N, Taccone FS, Citerio G, et al. Neuroprotection in acute brain injury: an up-to-date review. *Crit Care*. 2015;19:186.
4. Warner DS, James ML, Laskowitz DT, Wijdicks EF. Translational research in acute central nervous system injury: lessons learned and the future. *JAMA Neurol*. 2014;71(10):1311–1318.
5. Rincon F, Mayer SA. The epidemiology of intracerebral hemorrhage in the United States from 1979 to 2008. *Neurocrit Care*. 2013;19(1):95–102.
6. Hemphill JC 3rd, Greenberg SM, Anderson CS, et al. Guidelines for the Management of Spontaneous Intracerebral Hemorrhage: A Guideline for Healthcare Professionals from the American Heart Association/American Stroke Association. *Stroke*. 2015;46(7):2032–2060.
7. Krishnamurthy K, Laskowitz DT. Cellular and molecular mechanisms of secondary neuronal injury following traumatic brain injury. In: Laskowitz D, Grant G, eds. *Translational Research in Traumatic Brain Injury*. Boca Raton, FL: CRC Press, Taylor and Francis Group; 2016:97–126.
8. Hanrahan F, Campbell M. Neuroinflammation. In: Laskowitz D, Grant G, eds. *Translational Research in Traumatic Brain Injury*. Boca Raton, FL: 2016:127–144.
9. Shichita T, Ito M, Yoshimura A. Post-ischemic inflammation regulates neural damage and protection. *Front Cell Neurosci*. 2014;8:319.
10. Mracsko E, Veltkamp R. Neuroinflammation after intracerebral hemorrhage. *Front Cell Neurosci*. 2014;8:388.
11. Weisgraber KH. Apolipoprotein E: structure-function relationships. *Adv Protein Chem*. 1994;45:249–302.
12. Woo D, Kaushal R, Chakraborty R, et al. Association of apolipoprotein E4 and haplotypes of the apolipoprotein E gene with lobar intracerebral hemorrhage. *Stroke*. 2005;36(9):1874–1879.
13. Laskowitz DT, Horsburgh K, Roses AD. Apolipoprotein E and the CNS response to injury. *J Cereb Blood Flow Metab*. 1998;18(5):465–471.
14. Duan RS, Chen Z, Dou YC, et al. Apolipoprotein E deficiency increased microglial activation/CCR3 expression and hippocampal damage in kainic acid exposed mice. *Exp Neurol*. 2006;202(2):373–380.
15. Sheng H, Laskowitz DT, Mackensen GB, Kudo M, Pearlstein RD, Warner DS. Apolipoprotein E deficiency worsens outcome from global cerebral ischemia in the mouse. *Stroke*. 1999;30(5):1118–1124.
16. Aono M, Bennett ER, Kim KS, et al. Protective effect of apolipoprotein E-mimetic peptides on N-methyl-D-aspartate excitotoxicity in primary rat neuronal-glial cell cultures. *Neuroscience*. 2003;116(2):437–445.
17. Christensen DJ, Ohkubo N, Oddo J, et al. Apolipoprotein E and peptide mimetics modulate inflammation by binding the SET protein and activating protein phosphatase 2A. *J Immunol*. 2011;186(4):2535–2542.
18. Hoe HS, Pocivavsek A, Chakraborty G, et al. Apolipoprotein E receptor 2 interactions with the N-methyl-D-aspartate receptor. *J Biol Chem*. 2006;281(6):3425–3431.
19. Sheng Z, Prorok M, Brown BE, Castellino FJ. N-Methyl-D-aspartate receptor inhibition by an apolipoprotein E-derived peptide relies on low-density lipoprotein receptor-associated protein. *Neuropharmacology*. 2008;55(2):204–214.
20. Laskowitz DT, Thekdi AD, Thekdi SD, et al. Downregulation of microglial activation by apolipoprotein E and apoE-mimetic peptides. *Exp Neurol*. 2001;167(1):74–85.
21. Laskowitz DT, Goel S, Bennett ER, Matthew WD. Apolipoprotein E suppresses glial cell secretion of TNF alpha. *J Neuroimmunol*. 1997;76(1-2):70–74.
22. James ML, Sullivan PM, Lascola CD, Vitek MP, Laskowitz DT. Pharmacogenomic effects of apolipoprotein e on intracerebral hemorrhage. *Stroke*. 2009;40(2):632–639.
23. Laskowitz DT, Lei B, Dawson HN, et al. The apoE-mimetic peptide, COG1410, improves functional recovery in a murine model of intracerebral hemorrhage. *Neurocrit Care*. 2012;16(2):316–326.
24. Lynch JR, Wang H, Mace B, et al. A novel therapeutic derived from apolipoprotein E reduces brain inflammation and improves outcome after closed head injury. *Exp Neurol*. 2005;192(1):109–116.
25. Hoane MR, Pierce JL, Holland MA, et al. The novel apolipoprotein E-based peptide COG1410 improves sensorimotor performance and reduces injury magnitude following cortical contusion injury. *J Neurotrauma*. 2007;24(7):1108–1118.
26. Laskowitz DT, McKenna SE, Song P, et al. COG1410, a novel apolipoprotein E-based peptide, improves functional recovery in a murine model of traumatic brain injury. *J Neurotrauma*. 2007;24(7):1093–1107.
27. Hoane MR, Kaufman N, Vitek MP, McKenna SE. COG1410 improves cognitive performance and reduces cortical neuronal loss in the traumatically injured brain. *J Neurotrauma*. 2009;26(1):121–129.
28. Wang H, Anderson LG, Lascola CD, et al. ApolipoproteinE mimetic peptides improve outcome after focal ischemia. *Exp Neurol*. 2013;241:67–74.
29. Tukhovskaya EA, Yukin AY, Khokhlova ON, Murashev AN, Vitek MP. COG1410, a novel apolipoprotein-E mimetic, improves functional and morphological recovery in a rat model of focal brain ischemia. *J Neurosci Res*. 2009;87(3):677–682.
30. Wu Y, Pang J, Peng J, et al. An apoE-derived mimic peptide, COG1410, alleviates early brain injury via reducing apoptosis and neuroinflammation in a mouse model of subarachnoid hemorrhage. *Neurosci Lett*. 2016;627:92–99.
31. Gao J, Wang H, Sheng H, et al. A novel apoE-derived therapeutic reduces vasospasm and improves outcome in a murine model of subarachnoid hemorrhage. *Neurocrit Care*. 2006;4(1):25–31.
32. Wang R, Hong J, Lu M, et al. ApoE mimetic ameliorates motor deficit and tissue damage in rat spinal cord injury. *J Neurosci Res*. 2014;92(7):884–892.
33. Gu Z, Li F, Zhang YP, et al. Apolipoprotein E mimetic promotes functional and histological recovery in lysolecithin-induced spinal cord demyelination in mice. *J Neurol Neurophysiol*. 2013;2014(suppl 12):10.
34. Lei B, James ML, Liu J, et al. Neuroprotective pentapeptide CN-105 improves functional and histological outcomes in a murine model of intracerebral hemorrhage. *Sci Rep*. 2016;6:34834.
35. Vespa PM, Martin N, Zuccarello M, Awad I, Hanley DF. Surgical trials in intracerebral hemorrhage. *Stroke*. 2013;44(6 Suppl 1):S79–S82.
36. Mendelow AD, Gregson BA, Rowan EN, et al. Early surgery versus initial conservative treatment in patients with spontaneous supratentorial lobar intracerebral haematomas (STICH II): a randomised trial. *Lancet*. 2013;382(9890):397–408.
37. Maas MB, Rosenberg NF, Kosteva AR, et al. Surveillance neuroimaging and neurologic examinations affect care for intracerebral hemorrhage. *Neurology*. 2013;81(2):107–112.
38. Webb AJ, Ullman NL, Morgan TC, et al. Accuracy of the ABC/2 score for intracerebral hemorrhage: systematic review and analysis of MISTIE, CLEAR-IVH, and CLEAR III. *Stroke*. 2015;46(9):2470–2476.
39. Hemphill JC 3rd, Bonovich DC, Besmertis L, Manley GT, Johnston SC. The ICH score: a simple, reliable grading scale for intracerebral hemorrhage. *Stroke*. 2001;32(4):891–897.